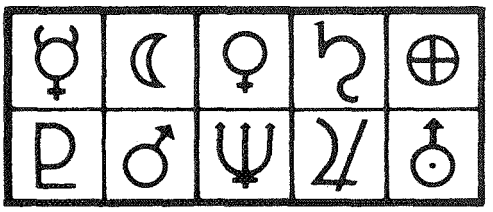

PLANETARY QUARANTINE

QR 17
June 1970

SANDIA LABORATORIES QUARTERLY REPORT
PLANETARY QUARANTINE PROGRAM

Prepared by:
Planetary Quarantine Department 1740

SANDIA LABORATORIES

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SANDIA LABORATORIES QUARTERLY REPORT - PLANETARY QUARANTINE PROGRAM

Seventeenth Quarterly Report of Progress

for

Period Ending June 30, 1970

Planetary Quarantine Department
Sandia Laboratories, Albuquerque, New Mexico

June 1970

Project No. 0064010

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SANDIA LABORATORIES QUARTERLY REPORT
PLANETARY QUARANTINE PROGRAM

Summary of Activities

Thermoradiation Sterilization. Experimental activities this quarter fell into three primary categories. First, further low temperature work was undertaken. Of particular significance was thermoradiation sterilization of B. subtilis var. niger spores at 60°C with simultaneous γ-radiation at 8 Krads/hr. The D value for this combined environment was found to be 6 hours. The D value in dry heat alone is in excess of 53 hours at 60°C. The second area of work was embedded spores. Spores were embedded in methyl-methacrylate and then exposed to thermoradiation environments at 105°C and varying dose rates. A synergism apparently comparable percentage-wise to that found for surface contamination was observed. Thus one D value for embedded spores at 105°C and 12 Krads/hr was essentially the same as the dry heat D value for exposed organisms at 105°C without the radiation added. Finally, brief studies were undertaken by other organizations at Sandia to investigate any deleterious effects of thermoradiation on typical electronic devices and materials and to determine the feasibility of building a relatively inexpensive thermoradiation chamber large enough to handle a Viking spacecraft.

Modeling of Thermoradiation Synergism. The thermoradiation inactivation model was used to generate a family of curves relating B. subtilis var. niger inactivation rates to temperature at given dose rates. It is shown how these curves may be used in planning sterilization cycles. A document

describing the modeling effort--including a physical rationale for spore inactivation in thermoradiative environments--was published this quarter.

Thermoradiation Inactivation in a Sealed Container. An attempt was made to simulate the behavior of embedded spores in thermoradiation environments by using small sealed aluminum containers. The inactivation of the spores in open containers behaved precisely like those on a surface. When the containers were sealed, results approximating those obtained from spores embedded in methylmethacrylate were obtained. The system is quite simple and offers hope of studying factors which might cause the high resistance of embedded spores. It also, once again, points out the pitfalls of studying spore inactivation in closed systems.

Studies on Bacterial Spore Inactivation. In continuing to study the mechanisms for bacterial spore inactivation in thermoradiation (and other) environments, it was found that there might be a link between the resistance of bacterial spores to alcohols and their resistance to heat. A preliminary investigation is reported here, but further study is required. If such a link is found, it offers a chemical means of investigating spore inactivation and variations in spore resistance.

Bacterial Spore Inactivation Modeling. Activity in this area during the past quarter has been directed toward the preparation of an interim report describing much of the past year's work. A brief summary of this document is presented here.

Humidity Control Systems. Two humidity control systems have been designed, built and tested during the past two quarters. These systems are capable

of controlling RH to ± 1 percent at ambient temperature and delivering this air to a temperature chamber. The RH in the system is monitored at ambient temperature from samples of air taken both before and after passing through the temperature chamber. One system is relatively portable but requires some periodic manual control. Hence, it is used primarily for short-term experimentation. The other system is slightly unwieldy but requires no manual control. Theoretically it is capable of long-term operation without adjustment or maintenance. Both are described in this quarterly report.

Bioburden Modeling and Experimentation. A document describing the basic bioburden model has been drafted this quarter. It describes the model's use for bioburden estimation, prediction and deriving some sampling requirements. In addition to this, computations involving variable fallout rates and removal factors have been begun. An example is presented here. Instrumentation to estimate a critical model parameter (numbers of organisms per "viable particle") has been developed and preliminary experimentation for model verification has been begun.

Computerized Identification System. A very general and versatile computerized identification system has been designed, programmed and tested against the PHS Apollo data. This system has as inputs all information relating to tests, their interpretations and possible organism categories. Consequently, it is easily modified to keep pace with the PHS improvements in their identification scheme. In terms of agreement with PHS identification, this system performs essentially the same as the less general prototype system used for feasibility studies described in QR 15.

Federal Standard 209a. At the request of the AEC, the status of Federal Standard 209a was reviewed. It was our opinion that no revision was currently needed.

Thermoradiation Sterilization

A. Description. The objective of this activity is to thoroughly investigate the sterilizing effects of combinations of heat and radiation, and to assess the practicality of this process for spacecraft sterilization. Thermoradiation offers the possibility of sterilization at temperatures less than 100°C at low dose rates of approximately 10 Krad/hr. This is possible because a synergistic effect in bacterial inactivation has been observed when combinations of heat and radiation are used simultaneously. Should any spacecraft components prove to be heat sensitive at high temperatures, thermoradiation offers a potential means of overcoming reliability problems.

There appears to be potentially significant spin-off possibilities for the thermoradiation process in the sterilization of drugs, pharmaceuticals, cosmetics and food. This is particularly true at the lower temperatures.

B. Progress. Activities this quarter have been concentrated in three major areas. These are described separately below.

1. Low Temperatures and Surface Contamination. A series of experiments was performed to obtain additional data at temperatures below 100°C. This low temperature data combined with the variable gamma irradiation dose rate data provides for further optimization of heat and radiation levels where lower temperatures are desired to achieve effective sterilization. Experimentatal results have shown that good synergistic effects are available as low as 60°C.

Figure 1 illustrates a typical D value versus dose rate curve for fixed temperature. Even at lower temperatures, we find that the curves are of this form with only an upward shift as the temperature decreases. The knee in the curve remains at about the same dose rate value of, roughly, 8 Krads/hr. Beyond this value, a unit increase in dose rate yields smaller and smaller returns in inactivation at all temperatures thus far investigated. Thus it seems of little value to use massive dose rates when potential side effects of radiation are a consideration. Accordingly, much of the experimentation this quarter was performed at a dose rate of 8 Krads/hr. This data is summarized in Figure 2 where it is shown that at 8 Krads/hr,

$$D_{105} = 1.5 \text{ hours}$$

$$D_{100} = 2.2 \text{ hours}$$

$$D_{90} = 3 \text{ hours}$$

$$D_{85} = 2.7 \text{ hours, and}$$

$$D_{60} = 6 \text{ hours.}$$

This data provides an opportunity to test the mathematical model developed to predict microbial inactivation during thermoradiation exposure. As can be seen from Figure 2, the model predictions are quite accurate. A more complete set of these curves for dose rates between 5 and 50 Krads/hr, as generated by the model, may be found elsewhere in this report.

Of particular interest this quarter was the inactivation of B. subtilis var. niger spores at 60°C with the 8 Krads/hr dose rate. This data is shown separately in Figure 3, and has particular relevance

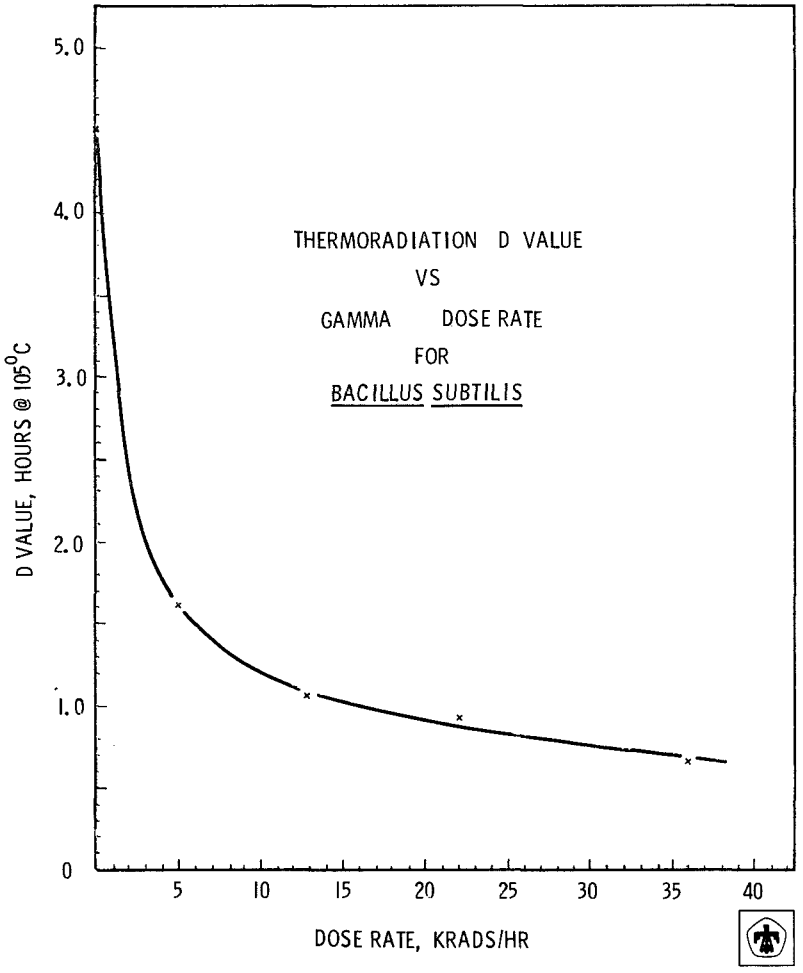


Figure 1.

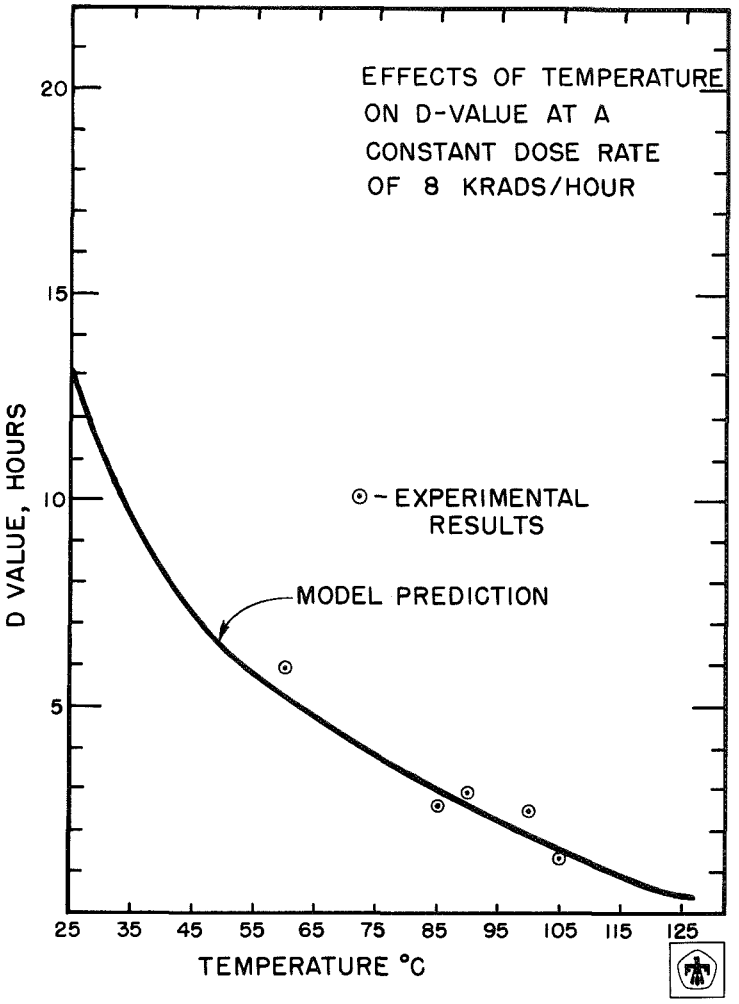


Figure 2.

because 60°C is the standard pasteurization temperature. Experimentation at the University of Minnesota indicates that dry heat D values for B. subtilis var. niger spores at 60°C will range from 53 to 274 hours depending upon the moisture conditions of the spores. With the addition of radiation at a rate of 8 Krads/hr (and 30 percent RH, ambient), this value becomes 6 hours (at worst a nine-fold improvement) computed from that data shown in Figure 3 using linear regression techniques. This D value for a complete experimental run at 60°C is slightly lower than that indicated by a preliminary run presented last quarter (QR 16). To obtain the same D value from dry heat alone would require a temperature in excess of 100°C.

Finally, Figure 4 shows survivor data at 95°C and 21 Krads/hr. The D values in this case is 1.5 hours--essentially the same as that for 105°C at 8 Krads/hr. These trade-offs are discussed briefly in the Thermoradiation Modeling section of this report.

2. Embedded Contamination. In recent years it has been found that the heat resistance of organisms is substantially increased when they are embedded in solid materials typical of potting compounds or casting resins. In particular, Angelotti, et al* found the dry heat D value for B. subtilis var. niger increased to 30 hours at 105°C in methylmethacrylate. For surface contamination the dry heat D value at 105°C is generally about 4.5 hours. With this data as a starting point we began

*"Influence of Spore Moisture Content on Dry Heat Resistance of Bacillus subtilis var. niger," Appl. Micro. 16(5): 741 (1968).

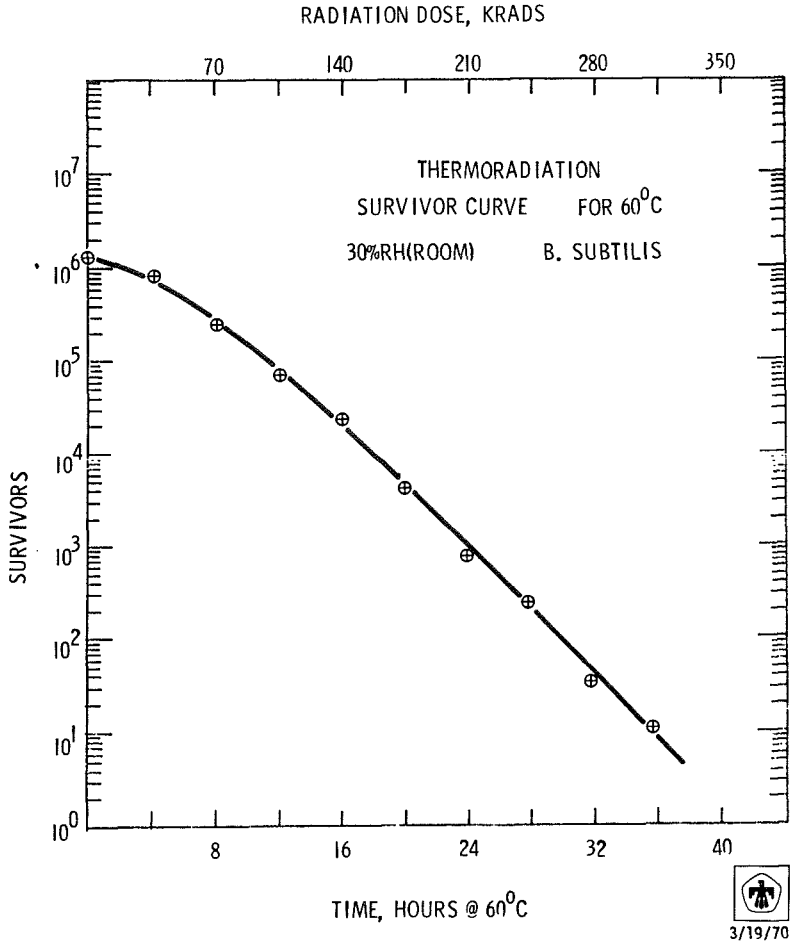


Figure 3.

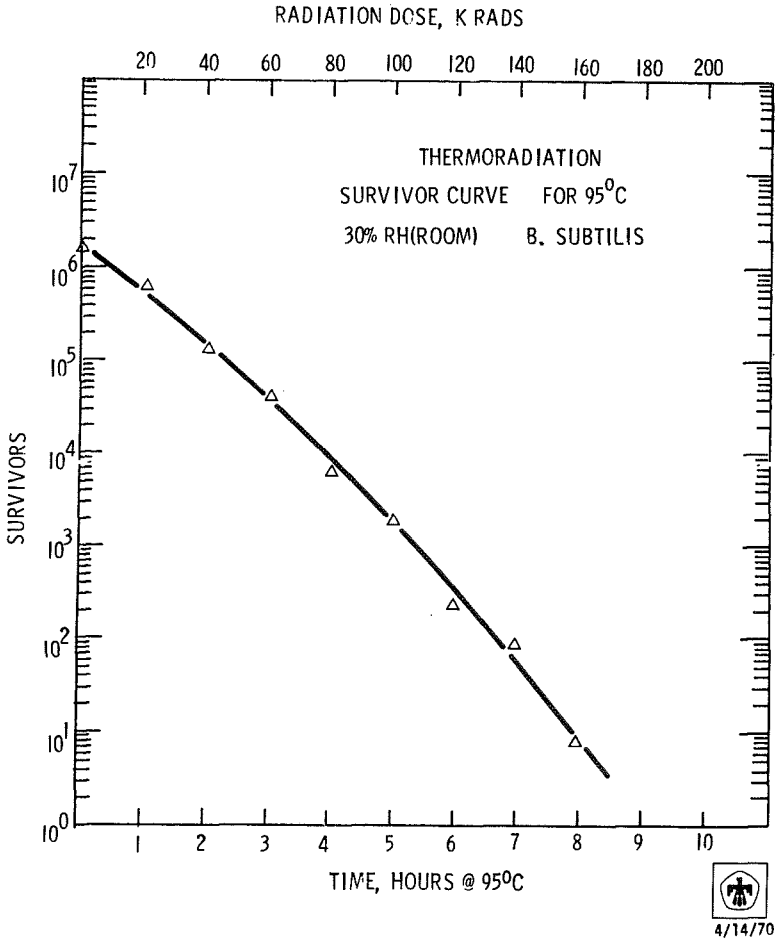


Figure 4.

an evaluation of the effectiveness of thermoradiation on embedded contamination.

To prepare the methylmethacrylate plastic with the embedded spores, the polymerization inhibitor was first removed from the liquid methylmethacrylate monomer. The liquid monomer was then mixed with spores in two different ways. The first means of mixing was to pipette two ml of a 10^9 ethanol spore suspension into a sterile beaker. After the ethanol had evaporated, the spores were equilibrated for 72 hours over a saturated solution of magnesium chloride ($MgCl_2$) resulting in a relative humidity of 33 percent at room temperature. Sixty ml of the prepared liquid methylmethacrylate monomer were then added to the beaker containing the spores. This mixture was insonated for two minutes at 12 watts per square inch to break up any spore clumps in the liquid monomer. Sixty grams of methylmethacrylate powder (polymer) were added to the liquid and the mixture was placed under vacuum and stirred with a magnetic stirrer. After 18 minutes of stirring and evacuation of air from the mixture, the methylmethacrylate was partially polymerized. This mixture was then poured out and pressed between plate glass sheets, to a thickness of 0.030 ± 0.001 inch. The glass sheets were sealed together and this assembly submerged in a water bath at a constant temperature of $50^{o}C$, for two hours and 45 minutes, for complete polymerization. The resulting sheets of plastic were hard, clear, free of bubbles, approximately 150 square inches in size and weighed about 100 grams.

Following Angelotti, et al, above, the second method used to inoculate the plastic with spores was to mix the ethanol spore suspension into the dry methylmethacrylate powder. The spore suspension was poured

over 30 grams of methylmethacrylate powder and mixed for 5 minutes on a magnetic stirrer. The mixture was allowed to air dry at room temperature until all the ethanol had evaporated and then stirred again until the spore inoculum was well distributed throughout the powder. The inoculated powder was then equilibrated over a saturated solution of $MgCl_2$ at room temperature for 72 hours. Thirty ml of the prepared methylmethacrylate liquid monomer were added to the powder mix and this mixture placed under vacuum and stirred with a magnetic stirrer. When air bubbles no longer emerged from the mixture, it was removed from the vacuum and heated for 30 seconds at $50^{o}C$, to initiate polymerization. The mixture was then returned to the vacuum chamber and evacuated and stirred until it was partially polymerized. The plastic was then cast between the plate glass sheets, polymerized, and the sample chips cut and stored in the same manner as described below.

In both cases a 5/8-inch diameter hole saw which had been ground to a very thin cutting edge was used to cut the sample chips from the plastic sheet. Average weight of these sample chips was 0.203 ± 0.005 grams and each chip contained about 1.2×10^5 viable spores. The loss of approximately one log of spores was due to the effects of polymerization. The prepared plastic chips were stored under refrigeration at $0^{o}C$ or less until prepared for exposure to the sterilizing environments.

To assemble the samples from both preparation methods for exposure, three of the chips were placed on an aluminum strip and secured in place with a narrow strip of autoclave tape. These assembled sample strips were maintained under refrigeration at less than $0^{o}C$ until exposed to the sterilizing environments.

Recovery of the spores from the methylmethacrylate chips was accomplished by first dissolving the chips in acetone in 180 ml wide-mouth dilution bottles. To assure high purity of the acetone used, it was filtered through a 0.45 micron pore-size filter. To thoroughly dissolve the chips in the shortest possible time necessitated some constant agitation of the acetone and chip. This was performed by affixing the dilution bottles on a shaker table. It also necessitated placing the chips in metal baskets to prevent them from becoming attached to the inside of the bottle. The baskets were fabricated from perforated stainless steel sheets in a size to fit into the wide-mouth dilution bottles. With the plastic chips in the basket in 100 ml of acetone in the dilution bottle on a shaker table with constant agitation, complete dissolution was accomplished in about 2 hours. This time, however, varied somewhat with the time the chips were exposed to the gamma irradiation with some insoluble crystals evident with increased irradiation exposure. After dissolution, tenfold serial dilutions of the solution were prepared, as required, with sterile acetone. These dilutions were pipetted onto Gelman Metricel, alpha 6, sterile filters in a filter holder apparatus and vacuum filtered. The filtered spores were then washed with sterile acetone to rinse off any methylmethacrylate residue, followed by two sterile water rinses to remove the acetone. The filter containing the spores was then placed on a Trypticase Soy Agar underlay in a petri dish and incubated. The plates were counted after 24 hours of incubation at $35^{o}C$.

The two methods of embedding spores described above yielded different results. First of all, the latter method of inoculating the dry

polymer resulted in rather large clumps of spores in the final sample material. These clumps seemed to contain on the order of 10^3 spores each. The survivor curve, using this method is shown in Figure 5 and is labeled clumped spores. A linear regression of the data shown results in a D value of 2.5 hours. However, considering only that part of the curve with the minimum slope the D value would be about 6.5 hours. The second method of adding liquid monomer to dry spores resulted in significantly less clumping of spores. With the same temperature/dose rate conditions this method yielded an overall D value of 1.9 hours as shown also in Figure 5. Considering only the latter part of the curve the D value was 4 hours. The reason for the rapid initial decrease in population in both clumped and uniform cases has not been determined. It may be due in part to additional polymerization but a similar response was obtained using gelatine matrix equilibrated at 75 percent RH before exposure to thermoradiation treatment. Figure 6 is a comparison of two dose rates with spores uniformly distributed in the methylmethacrylate. These two curves are the first in an anticipated series for defining a D value versus dose rate relationship at $105^{o}C$ for methylmethacrylate. An increase in the dose rate from 12 to 30 Krads/hr reduced the D value for the portion of the curve with minimum slope from 5 to 4 hours.

3. Corollary Studies--In addition to the investigation of the sterilizating effect of thermoradiation, we have been concerned with two implications resulting from possible use of thermoradiation on spacecraft: The effects of thermoradiation on spacecraft components and materials and the facility requirements to accomplish spacecraft thermoradiation sterilization.

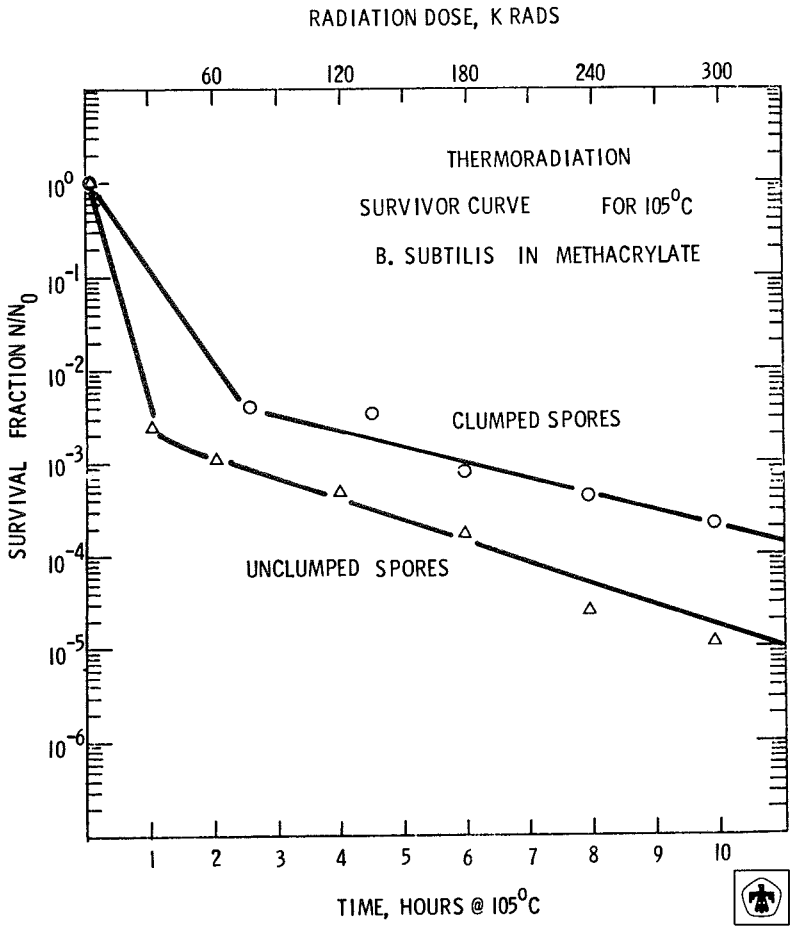


Figure 5.

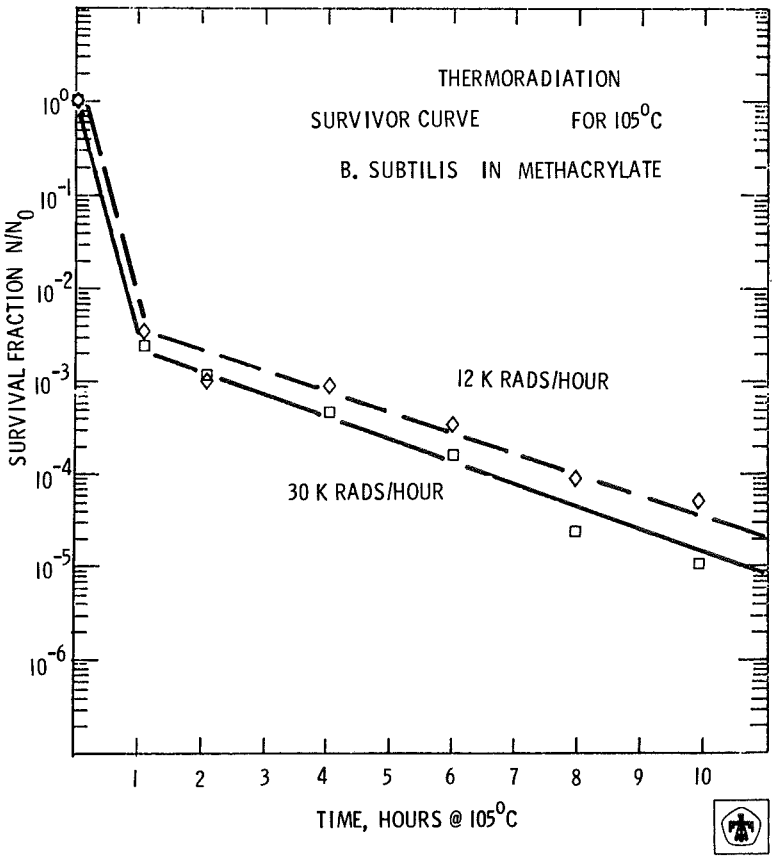


Figure 6.

A preliminary study was made by the Radiation Effects and Semiconductor Devices Department (2650) to evaluate the effects of thermoradiation on electronic components and spacecraft materials that might be affected. The study revealed no serious problems in electronic components provided they are designed or selected intelligently, with radiation in mind. Materials are not generally affected at the levels of radiation used. Teflon, one of the most radiation sensitive materials, exhibits little change after thermoradiation exposure. The addition of heat during the thermoradiation exposure tends to anneal the normal radiation damage to the teflon.

A study made by the Reactor Source Applications Division (5221) reviewed the facilities required to accomplish thermoradiation sterilization of an entire spacecraft. Based on the sterilization cycles we have developed at low temperatures of $80^{o}C$ to $105^{o}C$ and low irradiation levels of 10 Krads/hr, the facility requirements are entirely feasible. Estimated maximum additional cost is about $150,000 for the gamma facility.

Information on these two studies along with the thermoradiation process briefing was presented to a meeting attended by personnel from NASA space flight centers and contractors during the quarters.

A report titled "A Study of the Effectiveness of Thermoradiation Sterilization" was prepared and is in the final stages for publication as an SC-RR. This report updates the information on this subject as derived from the work performed since the last report, SC-RR-69-857, December, 1969.

Modeling of Thermoradiation Synergism

A. Description. The objectives of this activity are to develop a physically based model representing bacterial cell inactivation in composite environments of heat and ionizing radiation and to make this model available for analysis and for planning future work in this area. The model described is based upon the analysis of the experimental results presented elsewhere in this report and in the two previous quarterly reports. It is expected that such a model will not only provide a means for obtaining operational parameters for sterilization but also lend insight into thermoradiation sterilization possibly leading to further increases in synergistic activity.

B. Progress. The semi-empirical model

$$E\,[n(t)] = n(0)e^{-kt}, \tag{1}$$

$$k = k_T + k_S = \frac{KT}{h} e^{14.55}\, e^{-16890/T} + r_d^{218/T}\, e^{6.15}\, e^{-2775/T}, \tag{2}$$

where T is temperature in ^{o}Kelvin and r_d is radiation dose rate in kilorads per hour represents the operational portion of the model for simultaneous applications of dry-heat and γ-radiation to our own spore stocks. For applications, one typically has a set of operational criteria. These criteria describe the desired number of logs of population reduction from some estimate of the initial load, the maximum acceptable temperature, the maximum acceptable total dose, and some upper limit on the time which can be used for the sterilization process.

The maximum acceptable temperature and dose used for sterilization are normally based on a measure of required component reliability.

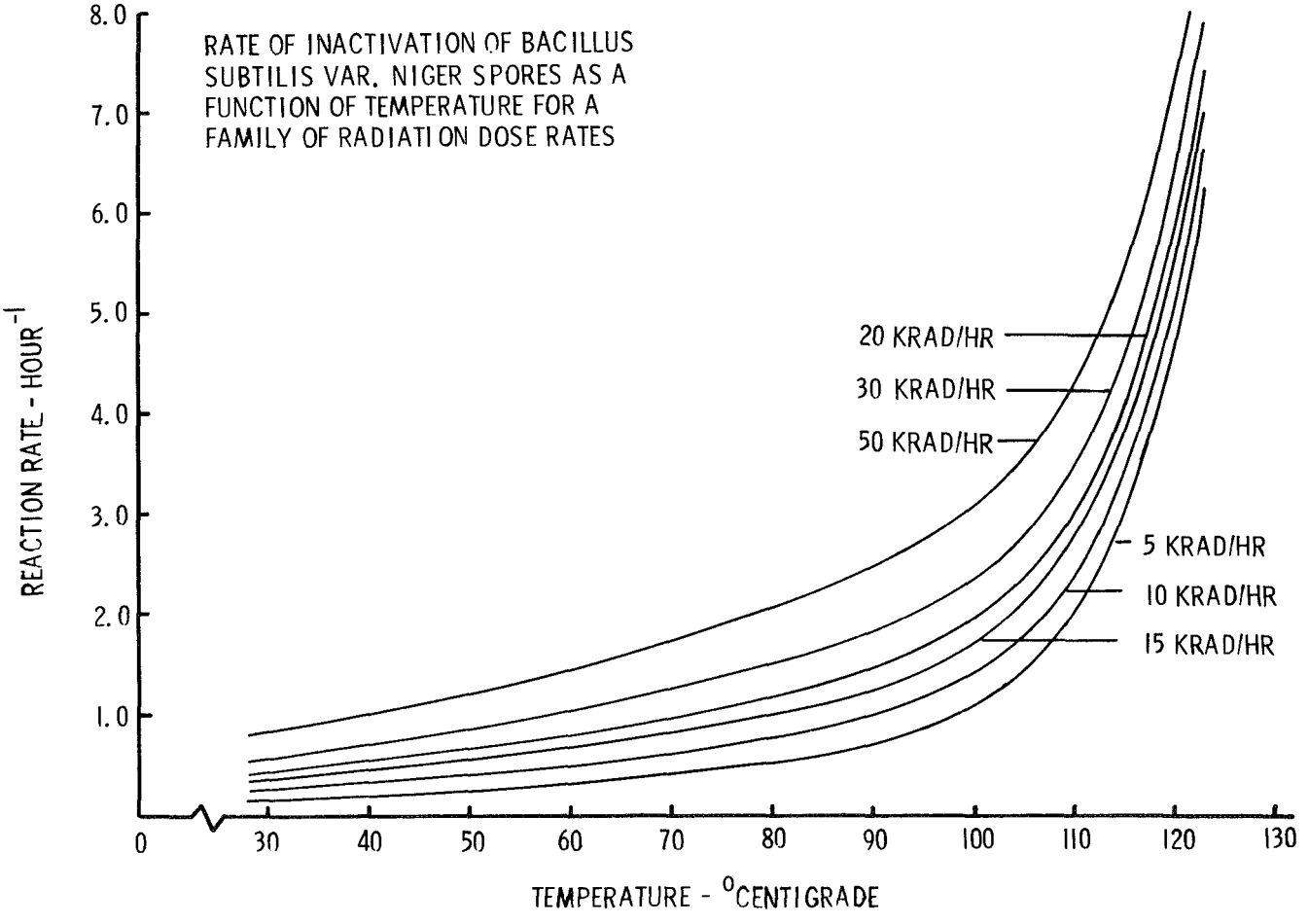
The family of curves shown in the accompanying figure are derived from the above model. These may be used in the definition of the sterilization process based on whatever criteria are stated. To see how this might be done, suppose the maximum allowable total dose and the upper limit in time, t_{max} (hours), for the sterilization process are prescribed, then the reaction rate required for n logs reduction in population is

$$k = \frac{1}{t_{max}} \ln\left(\frac{n(0)}{n(t_{max})}\right) \cong 2.3\left(\frac{n}{t_{max}}\right)$$

The maximum dose rate for the procedure is then equal to the total allowable dose divided by the maximum time, t_{max}, and the temperature T needed to provide the required reaction rate at the specified dose rate may be found from the accompanying family of curves by looking at the intersection of the reaction rate k with the curve for the maximum dose rate.

A document, "A Mathematical Model for the Thermoradiation Inactivation of Dry Bacillus subtilis var. niger Spores," was published this quarter (see Publications). This document essentially describes in detail the activities reported in the two previous quarterly reports (QR 15 and QR 16). The document includes a discussion of the original empirical derivation of the model, the analogy between the parameters in the empirical model and polymerization and the rederivation of the model from physical principles suggested by this analogy.

By way of spin-off, there is a strong suggestion that a model of this type would also be applicable to thermoradiation polymerization processes.



Thermoradiation Inactivation in a Sealed Container

A. Description. The objective of this course of experiments is to develop a practical experimental protocol which can be used to study the parameters affecting the rate of inactivation of microorganisms by thermoradiation in a closed, sealed system. In the course of this study it is hoped that at least a partial understanding of the decreased rate of inactivation in matrices such as methylmethacrylate, gelatin, epoxy, etc. will be obtained and that practical models of the situations can be derived.

B. Progress. The initial effort in this activity has been centered around the use of small aluminum ampoules. These ampoules were prepared by drilling 3/8-inch diameter holes 3/4-inch deep into 1-inch long sections of 1/2-inch aluminum bar stock. The mouth of each ampoule was threaded for a 1/4-inch long 3/8-inch x 24 socket head screw which was equipped with a tightly fitting 1/4-inch rubber "O" ring. The ampoules were arranged in groups of six each in small aluminum racks. Tests on the ampoules with the caps in place showed them capable of holding a minimum guage pressure of 30 psi.

The aluminum ampoules were loaded by placing 0.5 ml of 2×10^7 per ml ethanol suspension of Bacillus subtilis var. niger into the cavity and evaporating the ethanol into a 25 in. of Hg. vacuum for approximately 16 to 17 hours over a desiccant. Each set of six ampoules was then placed in the composite heat-radiation environment with three ampoules sealed with their caps and the other three ampoules open to the ambient

environment. After exposure the organisms were removed by depositing each ampoule in a cylindrical glass bottle filled with 25 ml of cold 1 percent peptone water and by sonicating the combination for 2 minutes.

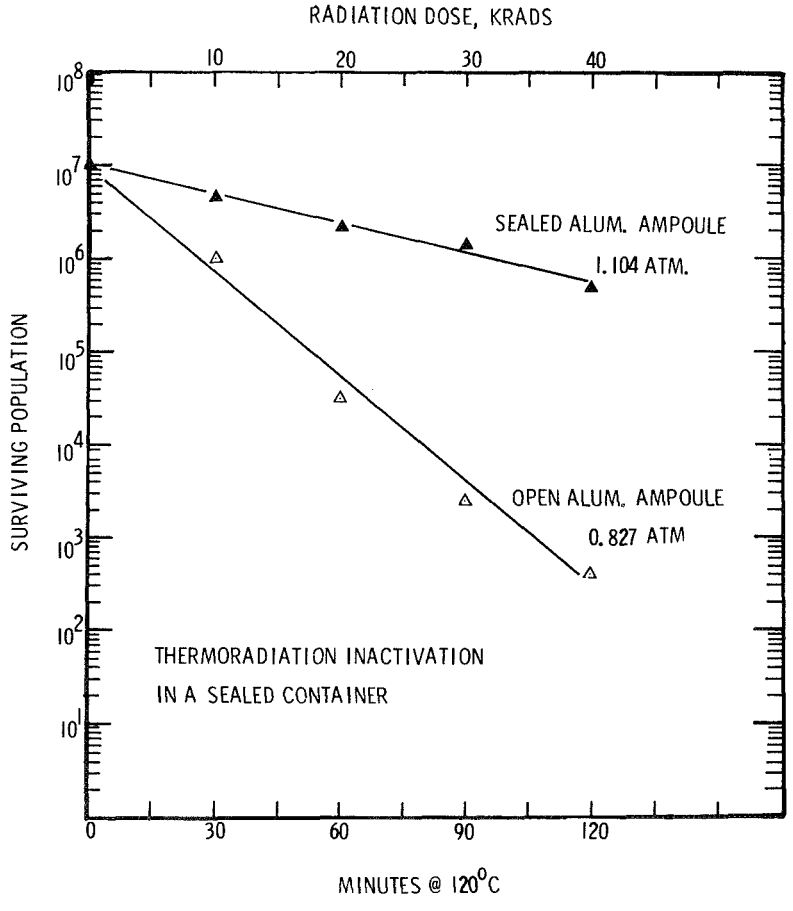
The rationale backing these experiments was to see if the microorganisms within the sealed system would have an inactivation rate significantly different from those within the open system as is observed for embedded organisms. Of particular interest is the pressure parameter since this parameter is increased in the sealed system due to the heating of its confined volume of air while the pressure of the open system remains at ambient.

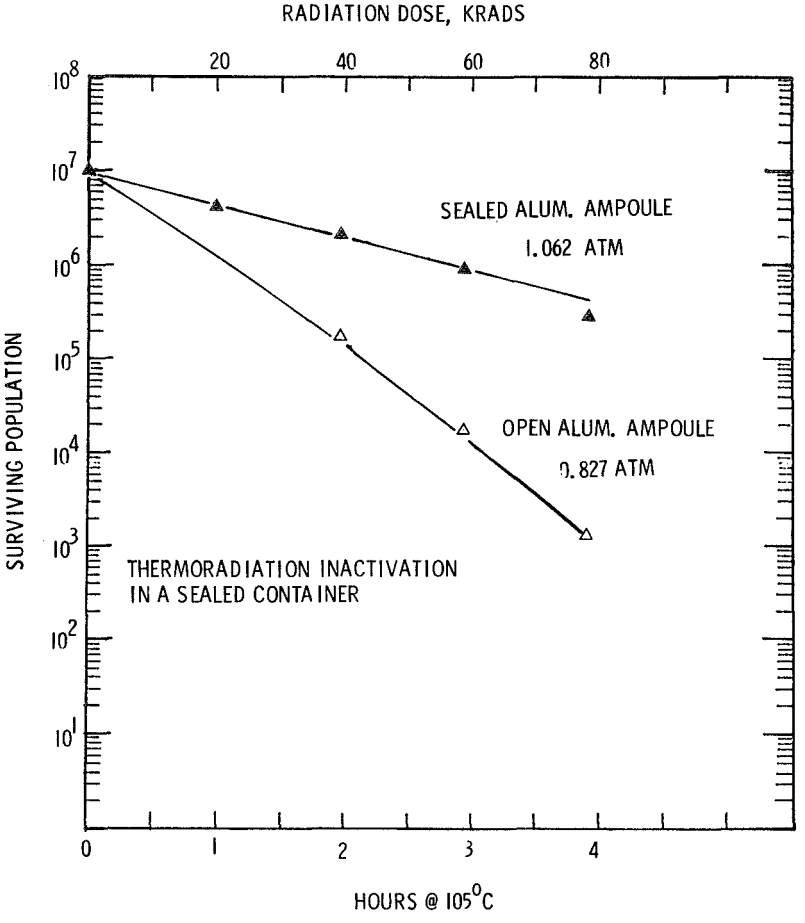
The following two figures of data represent the results obtained to date. The inactivation curves of the organisms at 105°C and 20 krads/hr show D values of 0.929 hours and 3.03 hours for the open and the sealed ampoules respectively. At 120°C and 20 krads/hr the D values were .44 hours and 1.64 hours for the open and sealed ampoules respectively. At 105°C the pressure within the sealed ampoule is approximately 1.062 atmospheres, and at 120°C the pressure is approximately 1.104 atmospheres relative to the ambient Albuquerque pressure of 0.827 atmospheres. Calcualted RH values for the systems are:

	Temperature	
	105°C	120°C
Open	0.45%	0.28%
Closed	0.59%	0.38%

accounting for atmospheric moisture. Possible moisture levels from other sources (outgassing, and so forth) are not known.

As can be seen the inactivation rate of the organisms in the sealed ampoules is considerably less than that of the open ampoules in each case. Of experimental importance is the fact that the D values of the inactivation curves for the open ampoules are the same as that obtained for similar temperatures and radiation dose rates using the standard experimental set-up of the thermoradiation study. Since spore inactivation in methylmethacrylate yields non-logarithmic curves, and has, to date been run only at 12 and 30 Krads/hr, no certain comparison can be made between the ampoules and plastics. Nevertheless, it would seem that the 3-hour D value at 105°C and 20 Krads/hr in the aluminum ampoules compares favorably with the 4-hour D value computed from the tail of the methylmethacrylate data at 105°C and a lower dose rate of 12 Krads/hr.




RADIATION DOSE, KRADS
20
40
60
80
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
SURVIVING POPULATION
SEALED ALUM. AMPOULE
1.062 ATM
OPEN ALUM. AMPOULE
0.827 ATM
THERMORADIATION INACTIVATION
IN A SEALED CONTAINER
0
1
2
3
4
HOURS @ 105°C

Studies on Bacterial Spore Inactivation

A. Description. One of the major problem areas relating to spacecraft sterilization is that of the variable resistance of spores to a given sterilant. For example, it is often observed that daughter stocks derived from wild type spores in a laboratory do not have their parent's extreme resistance to heat. Similarly different spore stocks of the same spore strain whether laboratory or wild type often have different resistance characteristics. As yet there is no way to confidently predict the resistance of randomly chosen spores--or even guess how high a resistance is possible.

Since spacecraft sterilization will probably involve the inactivation of random intramural bacterial spores of, possibly, many different species and resistance levels, a means of estimating these resistance levels is needed. Clearly the best way to resolve this problem is through a better understanding of why spores are resistant to the various sterilants that may be used in spacecraft sterilization. This approach offers the opportunity for ultimately assessing the likelihood that the D value chosen for sterilization is reasonable for the expected spore population of the spacecraft.

A number of approaches may be taken to studying the high resistance of spores. Initially, the most promising would appear to be a comparison between spores and their vegetative counterparts. For example, the fact that some species require dipicolinic acid for heat resistance, as demonstrated by H. Halvorson with B. cereus, was arrived at through the

observation that spores contain dipicolinic acid and vegetative cells do not.

B. Progress. Another potentially significant difference between some spores and their vegetative form was observed in the course of our work presented in QR 15 and QR 16. It has long been known that ethanol is bactericidal but not sporicidal. We have observed that ethanol in trace amounts prevents germination of B. subtilis spores. This effect is obviously a reversible one, since spores are often stored in ethanol. Still, ethanol is chemically active in spores as well as vegetative cells--but in a very different way. This raises the question: Given that ethanol is chemically active in spores is it possible that the resistance mechanisms of spores in some way prevent the ethanol from inactivating the spore. If so, this offers a potential means of chemically studying spore resistance. Preliminary efforts in this area have been started and are reported below. Basically, they demonstrate that there is an almost exact similarity between the gross properties of alcohols and their effectiveness as sporulation inhibitors and bactericides--the former being reversible, the latter not.

The process of spore germination can be followed by observing the changes in optical density for a spore suspension as a function of time (QR 15,16). Figure 1 illustrates the data obtained when spores of Bacillus subtilis var. niger were exposed to germinating media in the absence and presence of ethanol. Increasing the level of alcohol in the germinating media caused a decrease in the extent of spore germination. Such a result was observed for all the additives studied and presented in this investigation.

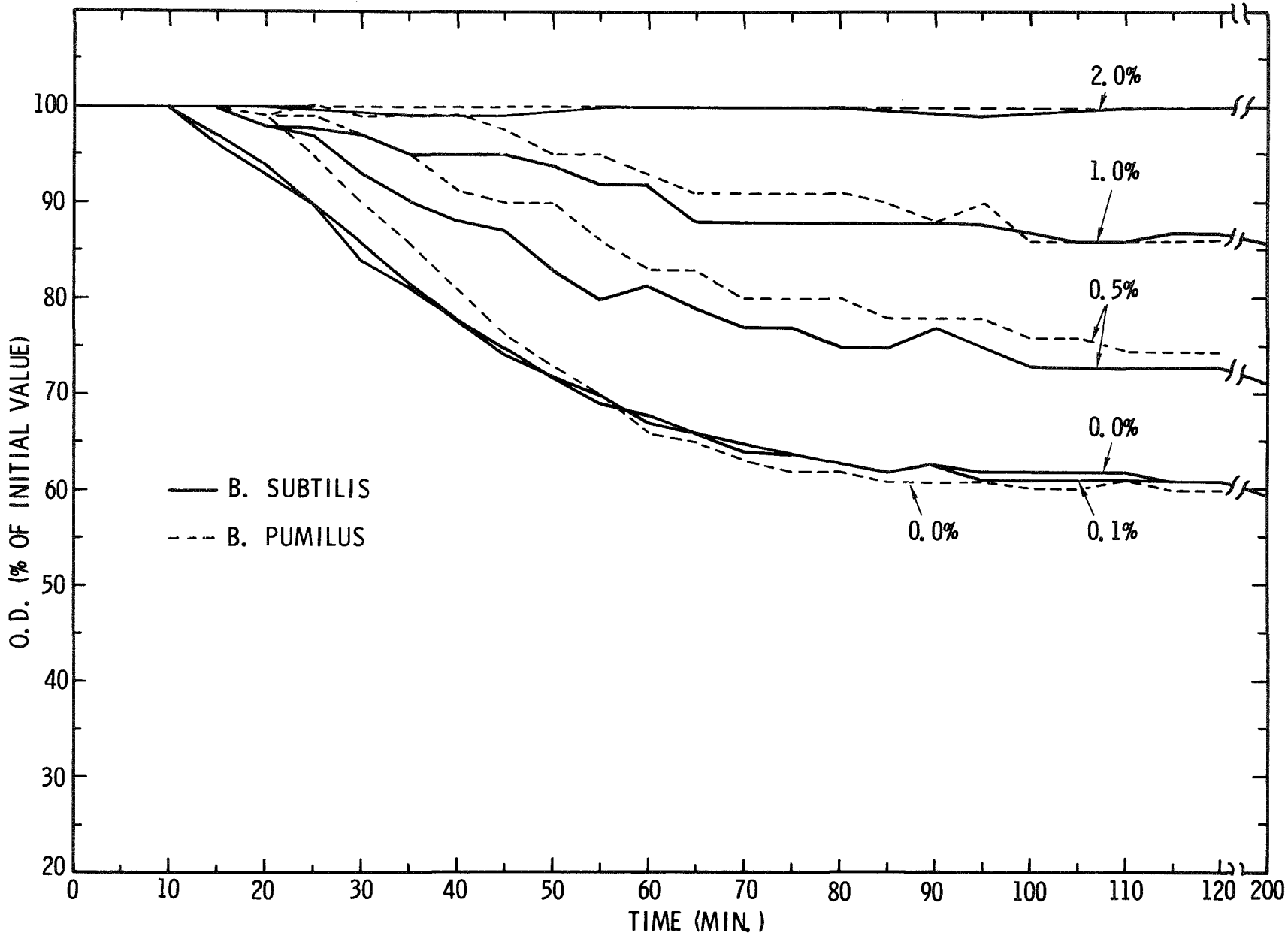

2.0%
1.0%
0.5%
0.0%
0.0%
0.1%
B. SUBTILIS
B. PUMILUS
O.D. (% OF INITIAL VALUE)
TIME (MIN.)
100
90
80
70
60
50
40
30
20
0
10
20
30
40
50
60
70
80
90
100
110
120
200

Figure 1.

Figure 1 also presents the germination data for Bacillus pumilus spores. It is of interest to note that the final extent of germination for both species is nearly identical, at a given ethanol concentration, while the shape of the germination curve for each species is different. The B. pumilus spores showed a lag prior to the initiation of germination when in the presence of alcohol.

A plot of the extent of germination as a function of alcohol concentration is presented in Figure 2. From such data it was possible to obtain an extrapolated value for the level of a given alcohol required to completely inhibit germination. Figure 3 illustrates the relationship between the alcohol concentration required for the total inhibition of spore germination and alcohol molecular weight. Analogs of n-propanol were used to study both the effect of alcohol structure and the effect of functional substituent on the extent of germination. 2-propanol was found to be a less effective inhibitor of spore germination than was n-propanol. Acetone, the ketone analog of 2-propanol, was found to be a less effective inhibitor of spore germination than 2-propanol. This data is also presented in Figure 3.

The spore suspensions containing the various alcohol concentrations, for example see Figure 1, were serially diluted and plated on Trypticase Soy Agar. The colony counts obtained from the various spore suspensions were essentially the same regardless of the alcohol, or the concentration of the alcohol, to which the spores had been exposed. Apparently, the inhibition of spore germination by alcohols could be reversed by the effective removal of the alcohol from the spore environment by the serial dilution procedure. The reversibility of the

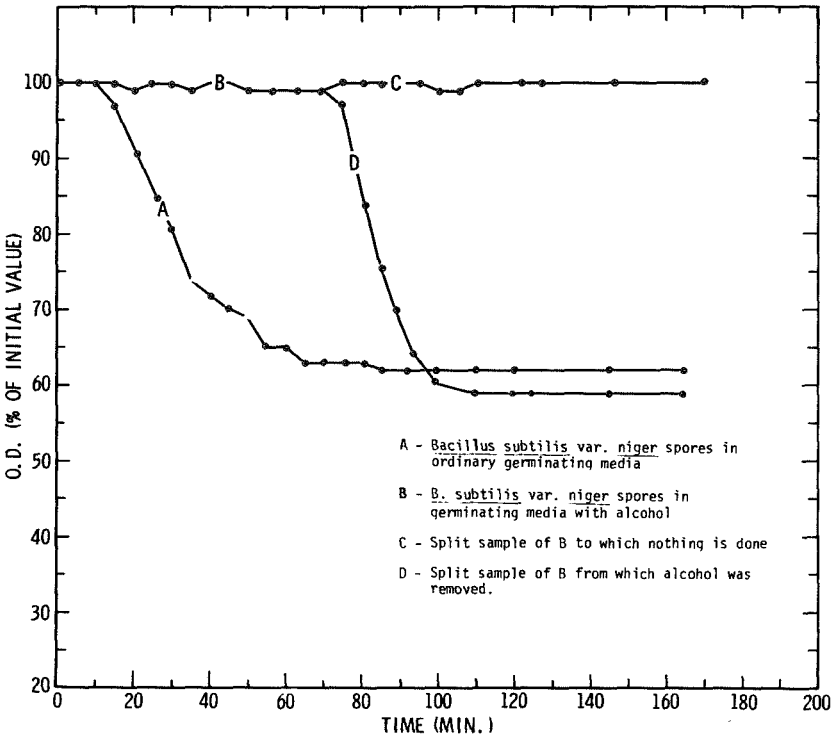


Figure 3.

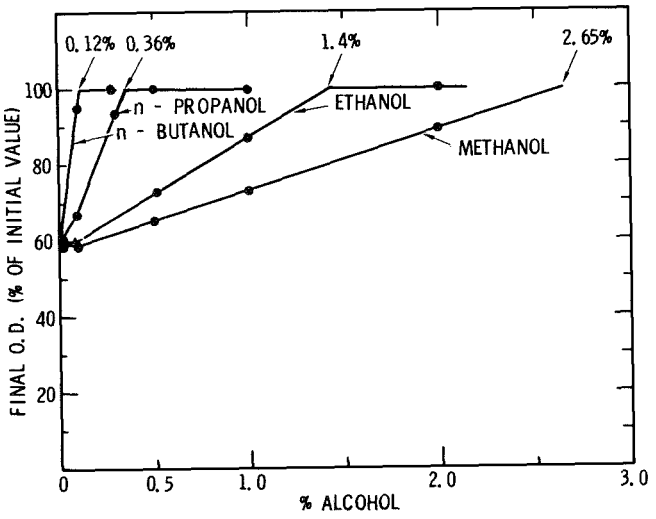


Figure 2.

inhibition of spore germination by alcohols is illustrated in Figure 4. The non-germinated spores were removed from the alcohol environment by Millipore filtration and re-suspended in Trypticase Soy Broth free of any inhibitory additive. The re-suspended spores showed normal germination.

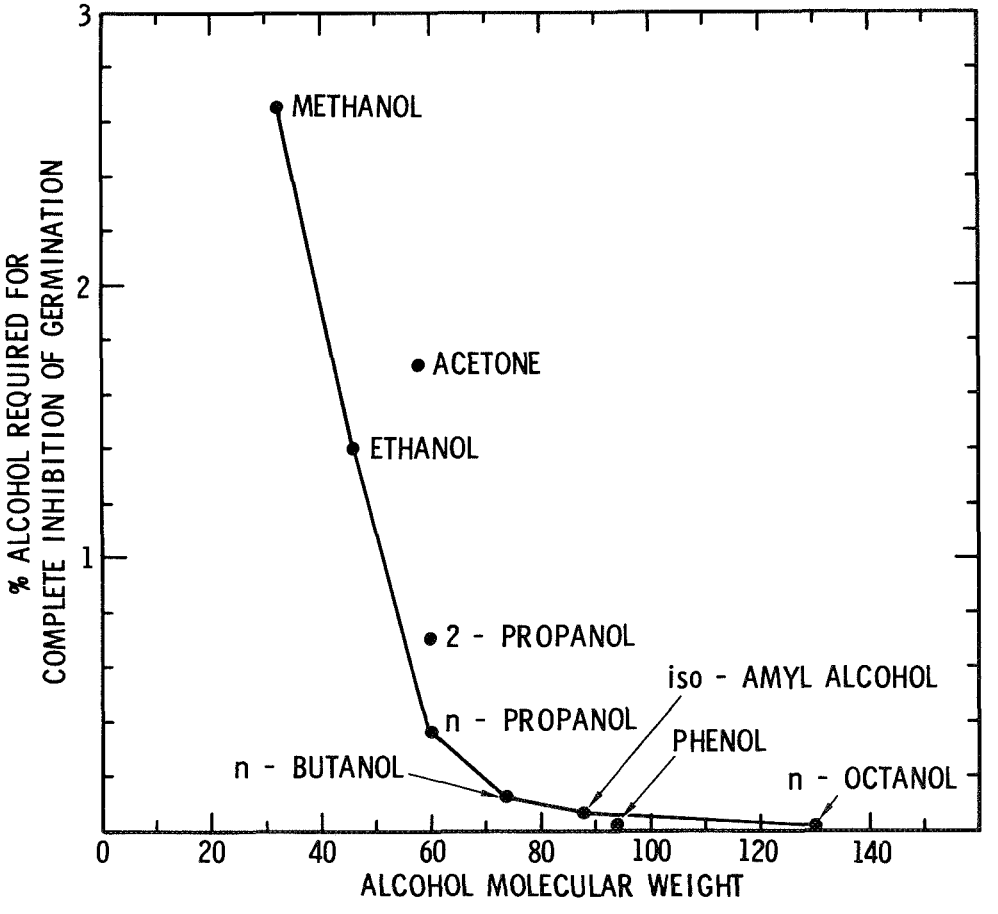


Figure 4.

The effect of alcohols on spores has not been extensively studied, perhaps due to the early work which established the non-sporicidal action of alcohols. However, some studies on the effect of alcohols on the development of dormant spores to vegetative cells have been undertaken. Some studies have shown that certain alcohols, i.e., 2-phenylethanol, chlorocresol, mixed esters of p-hydroxybenzoic acid, ethanol, and n-octanol, can inhibit the process of spore germination. This investigation establishes that remarkably low concentrations of a wide variety of alcohols, aliphatic as well as aromatic, can totally inhibit spore germination. This inhibition appears to be completely reversible.

The activity of the alcohols in inhibiting spore germination increases as their molecular weights and chain lengths increase (Figure 3). This same relationship is observed for the action of alcohols as bacterial disinfectants. It is of interest that primary alcohols are more effective bacterial disinfectants than are secondary alcohols and in this study a primary alcohol, n-propanol, was more effective than a secondary alcohol, 2-propanol, in inhibiting the spore germination process. Such correlations between the effect of alcohol on bacterial vegetative cells and bacterial spores suggest that the mechanism of alcohol inactivation may be similar for both systems. A basic difference is that the inactivation of vegetative cells is non-reversible, while the inhibition of spore germination by alcohols appears to be completely reversible.

The aliphatic alcohols have been found to inhibit spore germination at remarkable low concentrations in this study. Curran and Knaysi*

*Curran, H. R. and Knaysi, G., J. Bacteriol. 82, 793 (1961).

reported nearly complete inhibition of B. subtilis spore germination by ethanol at 10 percent (v/v) and partial inhibition by octyl alcohol at 0.1 percent (v/v). Complete inhibition of B. subtilis spore germination was observed at 2 percent ethanol and 0.003 percent n-octanol in this study. The differences between the work of Curran and Knaysi and this investigation in defining the concentrations of alcohol required to inhibit germination are probably due to the differences in methods used to study spore germination. The concentration of n-octanol (0.003 percent) required to completely inhibit germination is an upper limit, since further study to more precisely define the level of n-octanol needed for inhibition was not undertaken. Incidentally, such a study together with an investigation of even higher molecular weight alcohols could prove most interesting and may be of value to the food industry as a means of controlling the spore forming bacteria. In this regard, the observation that B. pumilus spores responded to treatment with ethanol in practically the same manner as did spores of B. subtilis (Figure 1) is of interest. The fact that two species of spores could be inhibited by alcohol suggests that the inhibition of spore germination by alcohols may have general applicability to aerobic spores. A study to determine the capacity of alcohols to function as sporostatic agents could have very practical implications.

The similarities and major difference (reversibility) of the effects of alcohols on spores and vegetative cells might be explained in several ways, and these are now under investigation. It now appears possible to determine whether a spore's "resistance" to alcohols is directly related to its dry-heat resistance.

Bacterial Spore Inactivation Modeling

A. Description. One objective of this activity is to develop a physically based model of bacterial spore inactivation capable of exhibiting behavior paralleling all known forms of bacterial spore dry-heat survival data and consistent with known physical and chemical facts about spores. Clearly, the ultimate objective is the understanding of spore inactivation in dry-heat environments and some quantitative expression of this understanding for use in predicting the efficacy of sterilization cycles. Thus one phase of this activity has been to attempt to rationally incorporate environmental parameters to spore inactivation through the physical parameters occurring in the model.

B. Progress. The activity this past quarter has been devoted primarily to the preparation of an interim report describing the progress of the past year.

The first section of the report is devoted primarily to a discussion of the desirability of modeling and the basic assumptions upon which the modeling has been based.

The second section of the report describes the theoretical developments that have occurred in the past year. These fall basically into four categories.

1. Generalization of the Model--The model was expanded slightly to permit reaction types of the form

$$A \rightleftharpoons B \longrightarrow C$$

$$A \longrightarrow D$$

$$2E \longrightarrow F$$

as inactivating reaction types. The first two of these are assumed to be first order reactions competing to inactivate n_1 molecules of some (unknown) substrate A. The third is assumed to be an independent second order reaction which results in spore inactivation by inactivating n_2 of the assumed n_3 molecules of some (unknown) substrate E. Physical motivation is provided for the selection of this collection of reaction types in spore inactivation by heat.

Associated with each of the arrows above is a reaction rate constant k. There are five such parameters in the model. The model has been generalized so that each of these, in turn, is expressed in terms of parameters associated with the activation of the reaction according to absolute reaction rate theory. Specifically, each k is assumed to be of the form

$$k = \frac{KT}{h} e^{-(\Delta H_o^{\ddagger} - T\Delta S_o^{\ddagger} + p\Delta V^{\ddagger})/RT} \qquad (1)$$

where k is Boltzman's constant, h is Planck's constant, $\Delta H_o^{\ddagger}$ is the activation enthalpy of the reaction at zero pressure, $\Delta S_o^{\ddagger}$ is the activation entropy of the reaction at zero pressure, p is the pressure (at which the reaction takes place), $\Delta V^{\ddagger}$ is the activation volume of the reaction, R is the gas constant and T is the temperature at which the reaction takes place. Frequently this kinetic model is used with the

$p\Delta V^{\ddagger}$ term incorporated into $\Delta H_o^{\ddagger}$.

2. Incorporation of Water Activity--Data exist which show the behavior of the integral entropy and entropy of sorption for certain biomolecules as a function of water content or availability. Since the entropy of a molecule is, at any time, the sum of the entropies from all causes, it may be reasoned that the activation entropies of the above reaction types should have similar behavior patterns as a function of a_w. When this assumption is made, and $\Delta S_o^{\ddagger}$ is varied as a function of a_w in accordance with the experimental data, the resulting variation in survivors (computed using the kinetic model) has the same characteristic form as that observed in survivor studies as a function of a_w. While this evidence is indirect, it seems reasonable to incorporate water effects in dry-heat sterilization into the kinetic model through their effect upon the activation entropy of the assumed reactions. This is the approach taken in this document.

3. Incorporation of Pressure--Since pressure occurs explicitly as a parameter in Equation (2), the generalized kinetic model provides an easy means of incorporating pressure as an environmental parameter affecting bacterial spore inactivation in dry-heat environments. This approach provides information about both inactivation in a deep-space environment and in potting compounds. The effect of pressure depends highly upon the value assumed by the activation volume $\Delta V^{\ddagger}$, and only an order of magnitude estimate of its value is currently possible. Nevertheless, estimates made using the model and survivor data when compared with theoretical estimates are in agreement to within an order of magnitude. The numbers are quite large--between about 10 and 100 liter/mole--

suggesting that large molecules are involved in dry-heat sterilization.

4. Some Properties of DNA: Possible Implications in Inactivation--
In this section evidence is put forth that spore inactivation in moist environments is due to the inactivation of DNA. This evidence involves the use of the model and is circumstantial in nature. Additionally, it is shown how DNA, if acting as the critical substrate to be inactivated, may account for some non-logarithmic survivor curves.

Section Three of the document describes the many efforts at model verification that have been undertaken. Since the kinetic model contains a large number of parameters, it might be expected that the model will "fit" survivor data of all kinds. Indeed this is the case and offers little in the way of model verification. Verification efforts come under the three general headings described below.

1. Predictions as a Function of Temperature--Non-logarithmic survival data for several spore stocks--each at three temperatures--were obtained from the literature and in our own laboratory. Data at two temperatures were used to determine model parameter values. Then the model was used to predict survival at the third temperature. This prediction was then compared with the actual survival data at that temperature. Our laboratory stock was then subjected to a highly variable temperature profile between $65^{o}C$ and $135^{o}C$. Again the model was used to predict the survivor curve, which was then compared with survival data. The model predicted quite accurately in all these trials.

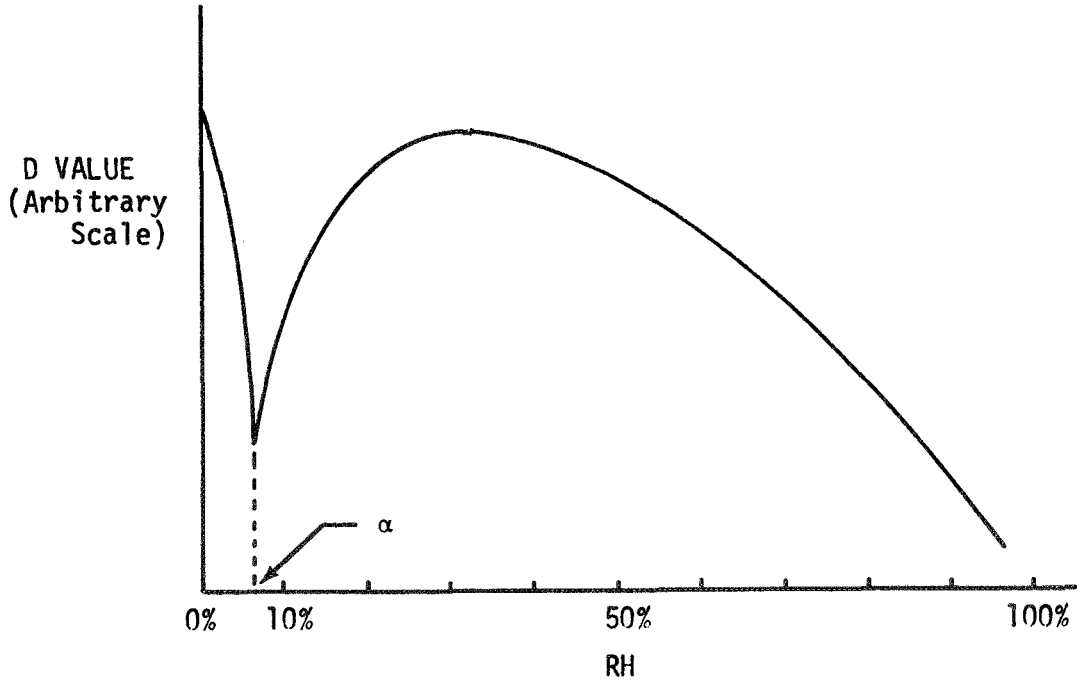
2. Reasonableness of Model Parameter Values--In the course of the survivor prediction experiments above, some values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were obtained ($p\Delta V^{\ddagger}$ being part of $\Delta H^{\ddagger}$). These values were compared with experimentally determined values of the same parameters associated with the denaturation of some RNA's, DNA's and enzymes. Model values were particularly compatible with some DNA data for the first order reactions and with some RNA data for the second order reaction.

3. Water Activity Predictions--Using water activity data provided by the FDA laboratories in Cincinnati, data at three temperature levels and one fixed baseline water level were used to determine model parameter values. Survival data at one temperature was used to determine $\Delta S^{\ddagger}$ as a function of water activity. Then the model was used to predict survival rates at the remaining two temperatures for water levels different from the baseline. The predictions were sufficiently accurate to lend credence to the validity of the model.

The last section of the document contains a complete mathematical derivation of the model and some additional approaches to modeling that might have applications to sterilants other than heat. In addition, a number of computational aspects of model verification are discussed.

Humidity Control Systems

A. Description. The relative humidity (RH) of the air in dry heat sterilization studies is known to have considerable effect on the heat sensitivity of microorganisms. Because of this, a method for closely controlling (and measuring) the RH of the air in experimental temperature chambers was considered essential. An additional reason exists for wishing to control RH precisely. It was observed in QR 13 that there are theoretical reasons for believing that the D-value depends on RH as shown below.



This is consistent with the experience of many investigators that "completely dry" spores are highly resistant and with that of other investigators in the RH range beyond, say, 10 percent. The range of

RH at less than 10 percent becomes important for two reasons if the curve is actually of the form shown. First, this is the RH range, at temperature, that a spacecraft will experience during dry-heat sterilization. Second, if the value of α (see figure) were known in this range--and if it were attainable practically during spacecraft sterilization--it would constitute the best choice of an RH value for dry-heat sterilization short of moving to very high RH values, which are impractical.

B. Progress. Two humidity control systems were developed this past quarter for the dry heat and thermoradiation sterilization studies. One system precisely mixes dry and moist air to achieve the desired RH. The other system saturates the air at a specific temperature which is controlled to a precise degree to provide the desired RH. Each of these is described separately below.

1. Air Mixing System--This system is depicted schematically in Figure 1 and consists basically of two channels of air flow, one for drying the air and one for saturation. The air supply normally used is the building pressurized air system with a separate regulator to maintain a maximum pressure between 10 and 15 psi as desired. The air flow through each channel is precisely metered through a tapered needle valve with a 1 cfm full flow meter providing a measure of the total air flow.

The air in the dry channel flows through a bed of desiccant in a sealed container and then on to the mixing chamber. The desiccant drys the air to less than 1 percent RH at room temperature.

The air in the other channel flows through fritted glass gas dispersion tubes submerged in water in a container which in turn is

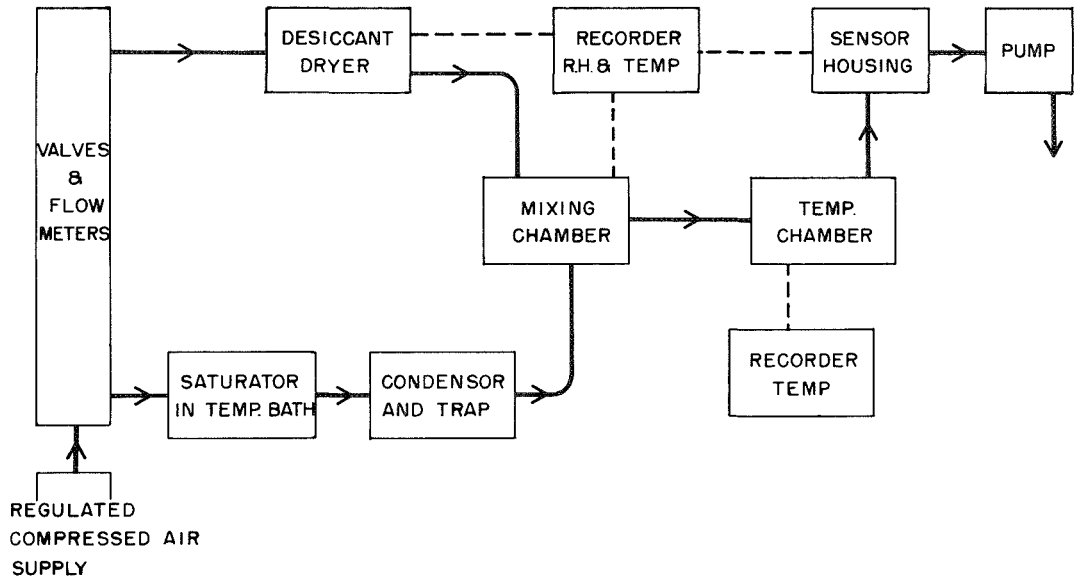


Figure 1. Humidity Control System A

partially submerged in a constant temperature water bath. The water bath is maintained at a temperature slightly above room ambient to assure a constant saturation temperature. The air then flows through a condensor coil of copper tubing suspended in the ambient air and into a small container to trap any condensed moisture. From the moisture trap the air flows into the mixing chamber.

The air from each channel is manually adjusted to provide the desired RH level in the mixing chamber as measured by a lithium chloride sensor. From the mixing chamber the air flows into the temperature chamber.

A small sample of air is drawn from the temperature chamber through a housing containing another lithium chloride sensor to measure the RH of the air at that point. A lithium chloride sensor is also located in the desiccant dryer chamber to measure the RH of the air coming out of the dryer.

This system is capable of providing RH control to less than ± 1 percent of the desired value as measured in the sample from the temperature chamber at ambient temperature. This degree of control can be maintained if all of the conditions affecting RH are maintained at reasonably constant levels. Since this is essentially a manual operation this system is used for relatively short-term experimentation.

Additionally, this system is made up of components of a size which are readily transportable and easily assembled and disassembled, making it ideal for relatively short-term studies at the Gamma Irradiation Facility located remotely from our laboratory.

2. Air Saturation at Controlled Temperature--The operation of this system is based on the premise that RH can be controlled in a system by controlling the temperature at which the air is completely saturated as shown by the chart in Figure 2.

The air supply used is normally the building pressurized air system with a separate regulator as in the previous system. The pressure can be adjusted as required to provide 1 to 2 cfm air flow with a metering valve for more precise control and a flow meter for measuring the volume of air flow.

The air flow is first directed through fritted glass gas dispersion tubes submerged in a container of water which is partially submerged in a constant temperature water bath maintained slightly above room ambient (about 26^{o}C). This provides good saturation of the air at a constant, set temperature.

The air then flows through a condensor coil of copper tubing and into a container to trap the condensed moisture. This condensor system

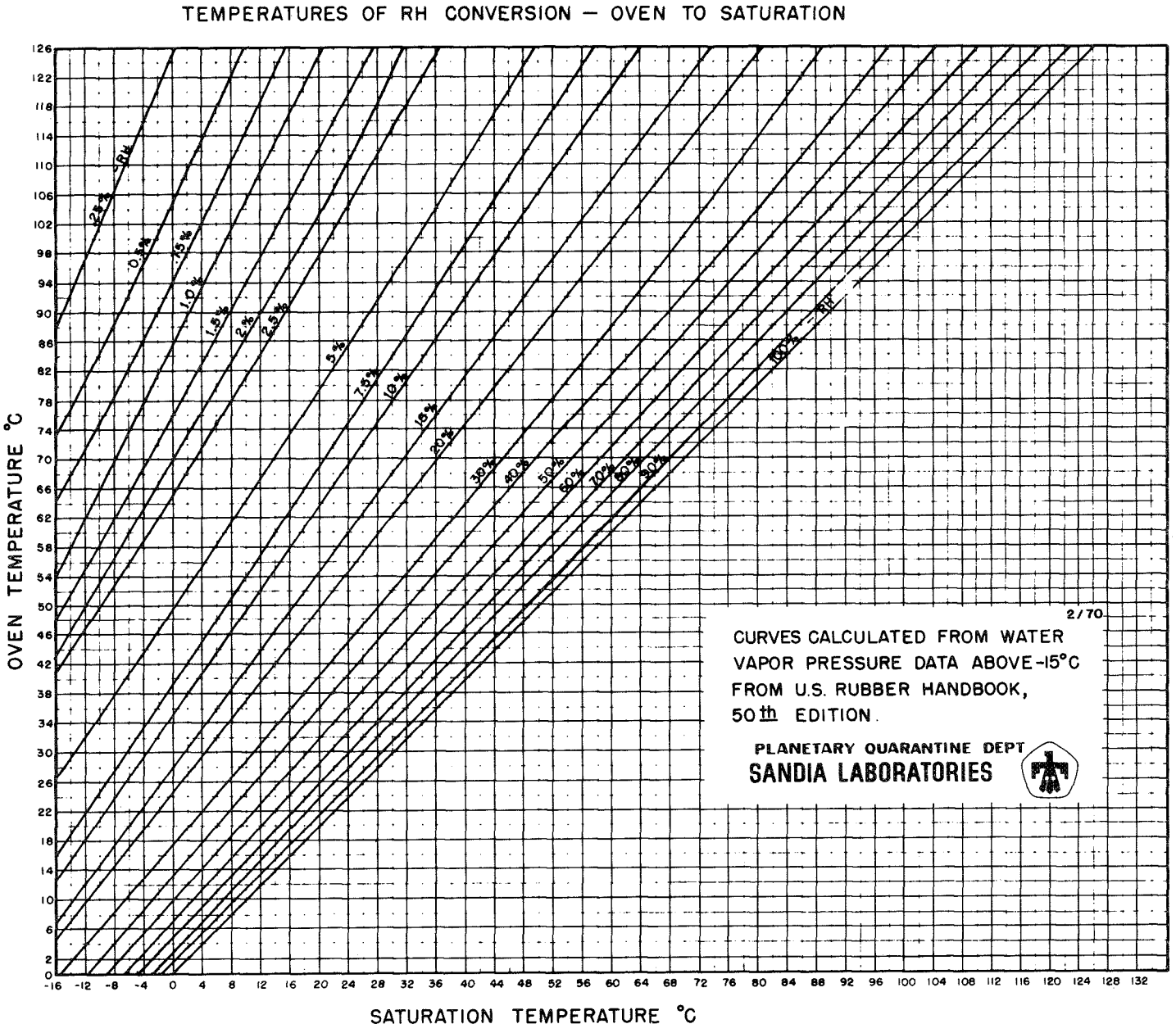

TEMPERATURES OF RH CONVERSION — OVEN TO SATURATION
OVEN TEMPERATURE °C
SATURATION TEMPERATURE °C
0.5%
1.0%
1.5%
2%
2.5%
5%
7.5%
10%
15%
20%
30%
40%
50%
60%
70%
80%
90%
100%
2/70
CURVES CALCULATED FROM WATER VAPOR PRESSURE DATA ABOVE -15°C FROM U.S. RUBBER HANDBOOK, 50th EDITION.
PLANETARY QUARANTINE DEPT
SANDIA LABORATORIES

Figure 2.

is submerged in a large volume (50 gallon) constant temperature liquid bath which can be maintained at temperatures down to $0^{o}C$ and lower depending on the liquid used and controlled to $\pm$ $0.1^{o}C$. Excess moisture in the air is removed in the condensor resulting in 100 percent saturation at the set temperature.

From the condensor the air flows through a coil and chamber located in the warm water bath. A lithium chloride sensor is located in the chamber to provide an RH measurement at the constant temperature. The air flow is then directed into the temperature chamber.

A sample of air is drawn from the temperature chamber through a coil in the warm water bath and through a chamber containing a lithium chloride sensor. This provides a RH measurement of the air sample at the same temperature as the RH measurement made of the input air to the temperature chamber.

A schematic of this RH control system is shown in Figure 3 and designated System B. This system is capable of controlling the RH to less than $\pm$ 1 percent of the desired value at room temperature. The system is not influenced by variations in the ambient conditions provided the complete air flow system is sealed to the ambient air. The size and bulk of the constant temperature liquid chambers makes this system less portable. Therefore, it is used primarily in the laboratory for long-term, dry-heat experiments.

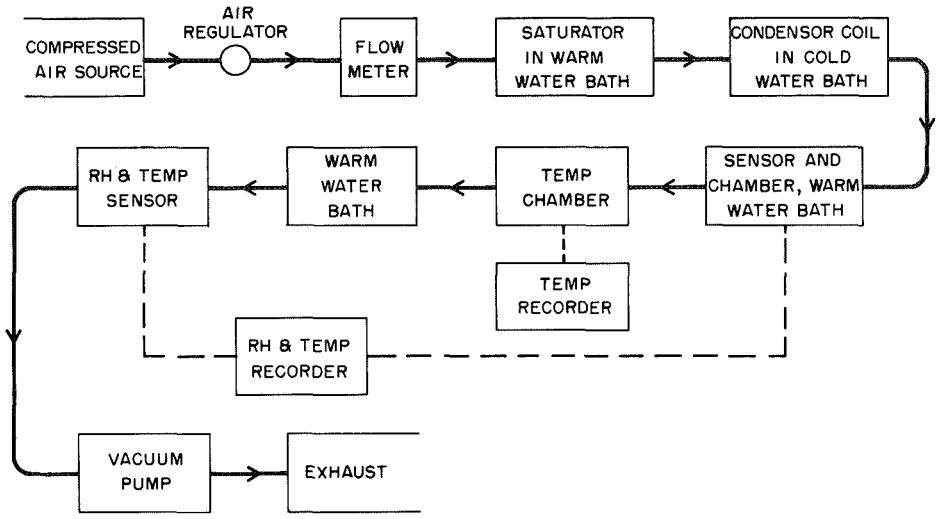


Figure 3. Humidity Control System B

Bioburden Modeling and Experimentation

A. Description. In attempting to estimate or predict the bioburden on a spacecraft surface at any time t, the first estimate usually obtained is that of the expected (or mean) bioburden H(t) at that time. It must be remembered, however, that the probability that the actual burden exceeds this estimate of H(t) can be quite large. Thus, in order to be confident about sterilization procedures, one needs more information about the bioburden than just an estimate of the mean burden. It would be desirable to have a means of answering questions of the form: What is the probability that the actual burden exceeds twice the estimate of H(t), three times the estimate of H(t), ten times the estimate of H(t), and so forth? The only hope of obtaining such information is to have explict knowledge of the quantities:

$P_j(t)$ = the probability that there are precisely j organisms on the surface of interest at time t, for j = 0, 1, 2, ...

Knowing this distribution of the number of organisms on the surface allows one to answer questions of the above form. Thus, for example, if the probability is sufficiently low that the actual burden exceeds ten times the estimate of H(t), sterilization cycles can be set for this ten-fold increase of the estimate knowing they will be in error with only a very small probability. Additionally, the choice of a two-, three-, or, say, ten-fold multiple of an estimated burden depends to some extent upon the number of samples taken in obtaining the estimate.

Thus, the more samples one takes, the more nearly the estimate of the expected bioburden coincides with the theoretical mean bioburden, thereby reducing the amount of subsequent compensation needed in answering questions of the above type. There is, therefore, some trade-off between this "compensating factor" (be it 2, 3 or 10) and numbers of samples. This trade-off can be analyzed only when the probabilities $P_j(t)$ are known--lending guidance for the establishment of sampling protocol.

From this, it seems reasonable that bioburden modeling, whether for estimation or prediction, should have as its aim the derivation of probabilities $P_j(t)$ representing the surface burden at time t. Parameters used to describe the $P_j(t)$ should be capable of being <u>estimated</u> from surface and/or environmental sampling data.

B. <u>Progress</u>. In the two preceding quarterly reports (QR 15,16) we have presented a bioburden estimation and prediction model which treats the changes in the bioburden on a surface as a stochastic process yielding a bioburden probability distribtuion $P_j(t)$ as discussed above. This model is summarized by the equations

$$P_j(t) = \text{Prob}\{j \text{ organisms on surface at time } t\} =$$

$$\sum_{j=0}^{\infty} \frac{e^{-H(t)/\gamma}(H(t))^k}{k!\,(\gamma)^k} \quad Q(j,k),\ j = 0,1,2,\ldots. \qquad (1)$$

The parameters in these equations are defined as follows:

$Q(j,k)$ = probability that k particles contain j organisms,

γ = mean number of organisms per particle

and,

$H(t)$ = mean number of organisms on the surface.

The parameters γ and $Q(j,k)$ can be estimated from a knowledge of the distribution of organisms per particle. Experimental and theoretical efforts are underway here, at Martin-Marietta, and the Public Health Service to gain such information. The remaining parameter, $H(t)$, is estimated directly from surface samples when Equation (1) is used as an estimation model. When Equation (1) is used as a prediction model, $H(t)$ is predicted using past surface data and environmental data. This latter can be done because $H(t)$ is related to the deposition rate of particles, $\lambda(t)$, and the removal fraction of particles, $\mu(t)$, through the differential equation

$$H'(t) = \gamma \cdot \lambda(t) - \mu(t)H(t) \qquad (2)$$

which can be solved for $H(t)$ as a function of γ, $\lambda(t)$ and $\mu(t)$.

Six activities have been undertaken during this quarter related to this model. These are discussed separately below.

1. We have discussed the model with Martin-Marietta in Denver. They are at present studying bioburden models as they will relate to the Viking Project. We have found many areas of joint interest and expect to pursue these in the future.

2. A document presenting a preliminary report on the model is in the final stages of preparation. This document will emphasize the compatibility of the sampling model and the prediction model, their utilization in predicting and estimating bioburdens based on surface samples and environmental samples, and the derivation of confident sterilization levels. Their use in establishing sampling protocols at contractor, project, and Planetary Quarantine Office levels will be the subject of a future document. These capabilities have been viewed as important

properties of bioburden models in a report by a study committee of the Planetary Quarantine Advisory Committee. We also attempt in this document to emphasize the "clumping" properties of the models and the resulting increase in the variance of the distribution, defined by the $P_j(t)$'s of Equation (1), over, say, the assumption of a simple Poisson distribution. Among other things, this implies a need for more samples to reach required confidence levels in estimates of mean bioburdens than would be required in the Poisson case.

This document, an interim report, will be published in the coming quarter.

3. In the last quarterly report (QR 16) we presented a technique for determining the deposition rate and removal fraction rate from surface sampling data. This allows us to modify our predictions on the basis of the samples taken from the surfaces. We illustrated this procedure on data gathered by the PHS at Cape Kennedy. In these examples we assumed a constant environment, that is, a constant removal rate and deposition fraction. During this quarter we have expanded our computer implementation to allow the deposition rate, $\lambda(t)$, and the removal fraction, $\mu(t)$, to vary as functions of time. This process will allow us to include in the model qualitative as well as quantitative information about the effects of different environments and activities on bioburdens.

Even though this study is not complete let us illustrate the type of results we are obtaining on the same data we used in QR 16. This data, kindly furnished by R. Puleo of the Spacecraft Bioassay Unit of the PHS, is shown below. It represents the results of sampling six

stainless steel strips each week beginning after the third week of exposure and continuing through the ninth week of exposure in Hanger AO at Cape Kennedy.

WEEKS OF EXPOSURE	MEAN NUMBER OF ORGANISMS PER STRIP
3	16.0
4	82.5
5	31.3
6	41.6
7	22.7
8	34.1
9	36.7

Consider first only the three data points corresponding to weeks 3, 4, and 5. This data is of particular interest for examining variable fallout rate and removal fraction because of its high peak at 4 weeks. We will again assume that $\mu(t)$, the removal fraction per week, is constant but let us assume this deposition rate has the form

$$\lambda(t) = a_0 + a_1 t + a_2 t^2 \qquad (3)$$

where we require $a_0 \geq 0$, and $a_2 \geq 0$. If we choose our coefficients μ, a_0, a_1, and a_2 to minimize the least square norm between sample data and H(t), as outlined in QR 16, we obtain

$$\mu = 0.773$$

and

$$\lambda = 4.89 + 1.41t + 0.t^2$$

These lead to a mean number of organisms, H(t), using Equation (2), illustrated by the solid line as a function of time in Figure 1. The broken line represents our mean obtained with $\mu(t)$ and $\lambda(t)$ both

constant (uniform environment assumption). We see little difference between these functions. Let us consider another example. With the form of $\lambda(t)$ given by Equation (3) and $\mu(t)$ is still assumed to be constant, let us consider all of the data given in the above table. Using all of the data and minimizing the least square norm between H(t) and the data, as per QR 16, we obtain

$$\mu = .914$$

$$\lambda = 8.85 + 0.t + 0.t^2$$

We see in this case we actually obtain a constant deposition rate, the coefficients of t and t^2 being zero. This is believed to be due to the type data we have. We observed in QR 16, the assumption of a uniform environment provides a good approximation for this data. These results substantiate this observation.

Figure 2 illustrates that this approach and the assumption of a constant environment seem to be compatible when the data warrants such an assumption.

4. Recently efforts to determine the model's sensitivity to variations in its parameters has been started. Preliminary results suggest that the model is relatively insensitive to reasonable variation in its parameters in ranges where they are expected to be, but the work is unfinished.

5. Further instrumentation has been developed to help investigate the distribution of the number of microorganisms per particle. In QR 16 we discussed a Sandia modification of Andersen samplers allowing extended sampling periods. This was used in parallel with a Royco

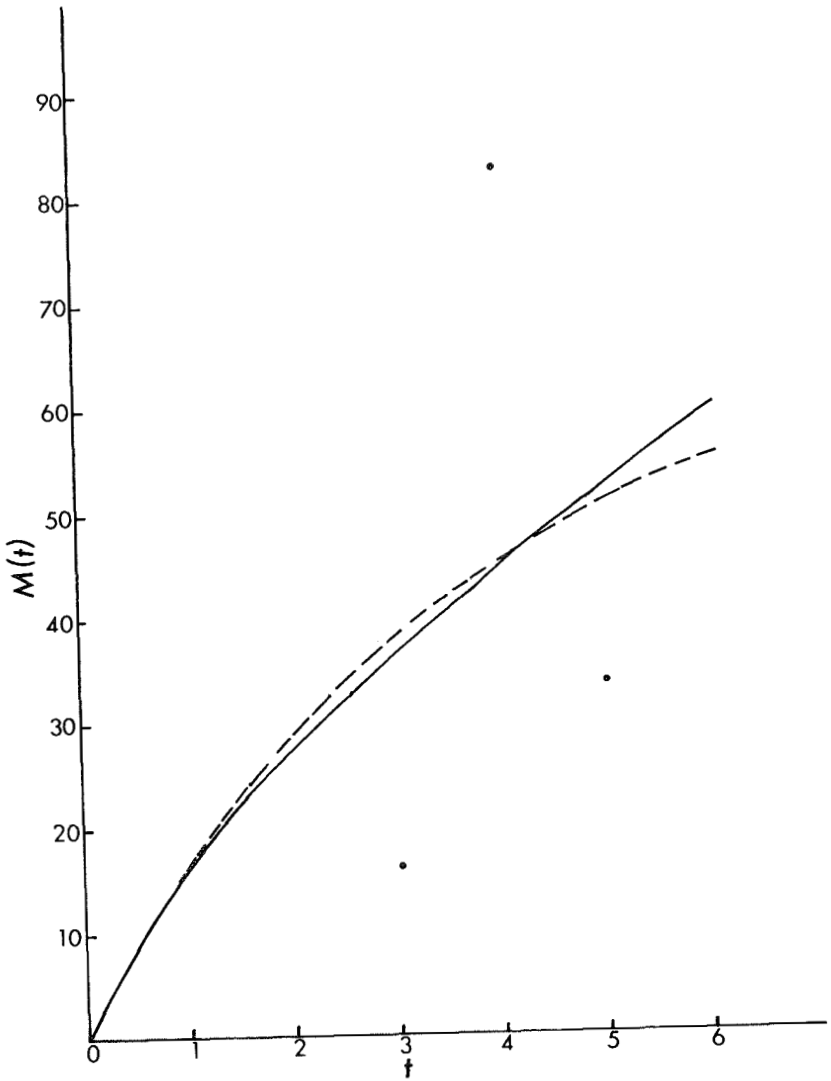


Figure 1.

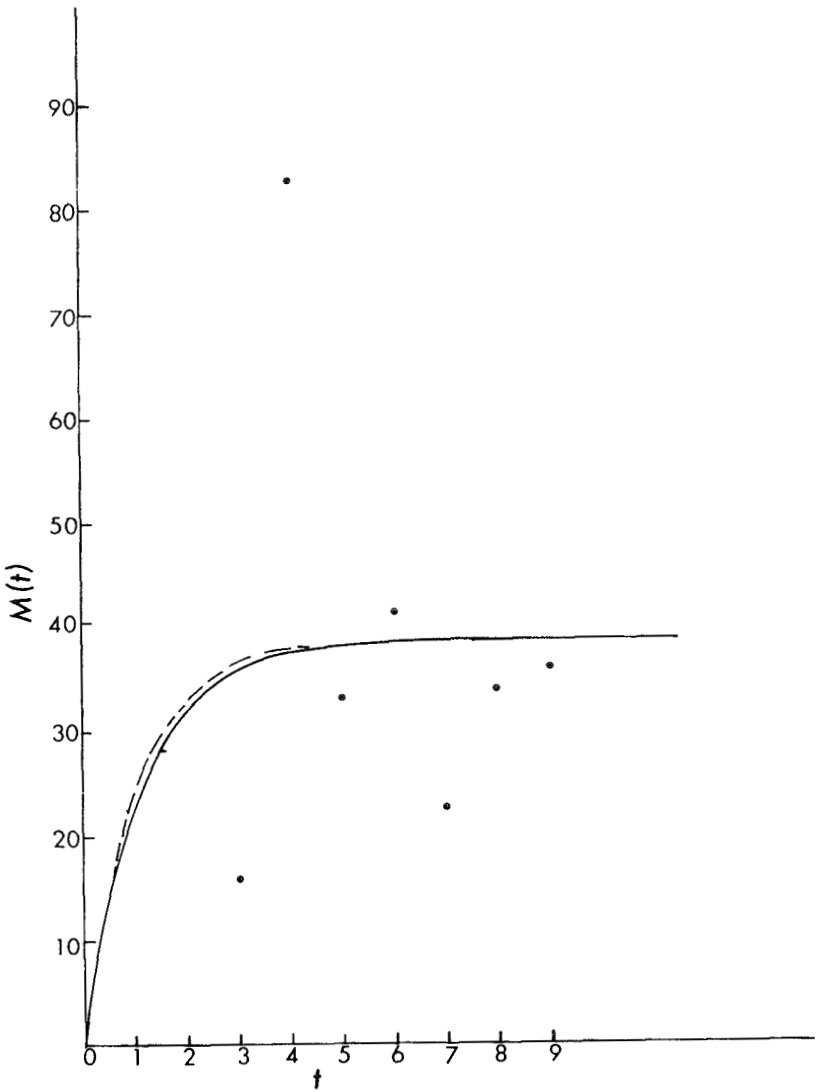


Figure 2.

particle counter to obtain a knowledge of the number of organisms per particle. This device is being used by the Spacecraft Bioassay Unit of the PHS at Cape Kennedy.

An additional electrical device has been designed and fabricated this quarter to shut down the above system when the total particle count, as determined by the Royco counter, reaches a predetermined level. In this way, the data will all relate to a fixed number of particles, and it will hopefully be easier to test certain hypotheses against it. For example, it has been suggested in the literature that the distribution of particles in droplets (conceivably an analagous process) is approximately Poisson. If the number of organisms per organism-bearing particle is approximately Poisson, then so also is the number of organisms for N organism-bearing particles (assuming the number on one particle does not affect that on another). Many tests of M particles (with M sufficiently large to get reasonable organism counts) can then be used to test whether the distribution of organisms per N organism-bearing particles is approximately Poisson; assuming, as seems reasonable, that N remains approximately the same when M, over which we have some control, is both large and fixed. If it is not, then the assumption of a Poisson distribution for the number of organisms per particle is likely not correct. If the converse is true, the Poisson distribution for the number of organisms per particle may not be the correct one, but the class of possible distributions is considerably limited.

In addition to this, a chemical which retards evaporation of water from Andersen plates was discussed in a recent meeting with K. R. May of the Microbiological Research Establishment, Wiltshire,

England. Using this chemical, oxyethylene docosanol, even in low relative humidities, the useful time of sampling may be extended to over 12 hours. Since it appears to have no effect itself upon organisms, the use of this chemical may be a way to avoid the dieoff of vegetative cells experienced with the sampling system at Cape Kennedy.

6. Efforts to experimentally investigate the validity of the bioburden model--both for estimation and prediction--have been begun. To investigate the model's validity, it is necessary to be able to determine independently in controlled experiments, the following items:

-- $\lambda(t)$, the particle deposition rate as a function of time

-- $\mu(t)$, the particle removal fraction as a function of time

-- the surface burden as a function of time, and

-- the distribution of the number of organisms per particle.

A laminar flow facility for simulating spacecraft assembly environments has been designed and reported on previously (QR 12). Also a means of tagging particles with organisms in a controlled manner has been devised and reported previously. Finally an acoustic particle dissemination device was developed (QR 10). This combined system is capable of maintaining a constant tagged particle deposition rate over a 6 ft^2 area ($\pm$ 10 percent from a mean value) for several days. The feasibility of determining an approximate distribution of the number of organisms per particle had been demonstrated. Clearly the bioburden on experimental surfaces in this facility can be determined.

The major stumbling block to model verification experimentation has been in the measurement of the removal fraction as a function of time. For model verification, any predictable or measurable particle

removal will suffice. This being the case, we have been attempting to find some means for removing particles from surfaces in a measurable fashion. Regardless of the method of removal, in order that the experiment be controlled, the percentage of particles removed from the surface by the air flow in the laminar flow facility must be reasonably constant. If measurable, the air flow may be used as the means of removal itself.

This quarter, the constancy of the removal of particles from surfaces by air flow in a laminar flow facility was investigated. It was found that, under even extreme circumstances, 10μ glass spheres (our experimental particles) could not be removed from small glass surfaces. Specifically, it was found that using factory-cleaned 22 mm square glass cover slips, aside from a few particles that came off initially, no particles were removed by 80 ft/min air flow in periods of up to five days. During short term tests, it was visually observed that very few particles were removed using air flow velocities up to 1000 ft/min. Since particle-to-surface adhesion forces can be reduced by first covering the glass cover slips with a monolayer of fine (less than 0.5μ) particles, and then loading the slide with glass spheres, this was tried. Arizona road dust proved most effective in reducing the particle-to-surface adhesion forces of all the fine particles used. The cover slips were prepared by rolling them in a container filled with Arizona road dust for 30 minutes. The cover slips were then removed, and using a Pasteur pipette connected with air under 30 psi pressure the cover slips were dusted free of excess particles. Microscopic observation showed that almost all of the remaining dust particles were less than 0.5μ in diameter. These cover slips were

then loaded with 10μ glass spheres and placed in an 80 ft/min air velocity. After a small initial change in particle loading, probably due to a few loose particles, no change in the number of particles was observed after a two-day period. In other words, particle removal due to air flow appeared negligible. At 1000 ft/min air velocities, by comparison, many of the 10μ glass particles were removed from the dust coated plates. Thus it seems certain that whatever means of controlled removal is found, the laminar air flow in the experimental facility will not interfere with its predictability.

Computerized Identification System

A. Description. The objective of this activity has been the development of a computerized version of the PHS microbiological identification system now being used at Cape Kennedy. If it agrees substantially with identifications now being made, such a program may be incorporated in the Lunar Information System to relieve the PHS of the actual identification of the colonies sampled during the outbound contamination inventory of the Apollo spacecraft.

B. Progress. The feasibility of modeling the identification process so that a computer could be used was demonstrated in QR 15 with data from Apollo 11. Agreement with the PHS identification was obtained with 83.7 percent of the colonies sampled on Apollo 11. Unfortunately the program to test feasibility of this concept used the output from QUALSUM which, itself, requires identified colonies as input. And, moreover, the program was written so as to continue the specific identification procedures then in use. As a result, it was extremely difficult to modify the program to keep up with improvements in the identification procedures. For incorporation in the Lunar Information System a program was needed that could use only the actual data, as recorded by the PHS, and that could be readily adapted to changes and improvements in the identification procedures. Ideally such a program would simulate only the decision process and have as input the particular test lists, their interpretation, and the microbial classifications that were desired. Such a program has been written and checked with the Apollo 12 data,

using the identification procedures and classifications used by the PHS at that time. The PHS procedures were modified beginning with Apollo 13 and these new schemes have also been run.

Ideally, an identification system consists of a set of tests whose outcomes define certain organism classifications. Practically it is more complicated due to the variability of test results for a given organism and also in part because of the desire to minimize the number of tests that must be done. The PHS identification system uses the results of five tests to determine further tests necessary to obtain the classification that they desire. Thus the first five tests determine a general grouping of organisms and a scheme specifies the further tests needed to identify the colony depending upon the outcome of the first five. Looking at the procedure from another point of view, ultimately, a series of test results are listed as pertaining to a sampled colony and from these must be deduced the correct organism classification. The decisions indicated by the first five tests as well as those in the individual schemes can be represented as matrices, which relate the tests and decision results to organisms classifications. This is illustrated below.

To simulate this identification process in as general a manner as possible, the decision matrices were read in as input to the program. Thus changes can easily be made in definitions of schemes, definitions of classification categories within schemes, identification tests and their results as well as the number of schemes, classification categories, or tests. To further simplify the simulation (and to allow the possibility of weighting test results in a convenient manner) the entries in the input matrices were one of +1 (positive), -1 (negative) or 99

(weak, variable, or inconclusive). These entries correspond in a general way to the usual interpretation given microbiological tests. Unfortunately some tests used by PHS have as many as nine possible outcomes as listed on the data sheet. Hence, it was necessary to write a transformation for each test so that its results would agree with one of the matrix entries above. Thus the test results can be interpreted by a microbiologist as usual. Being treated as input to the program these transformations can easily be modified as desired.

In addition to flexibility, the transformation-matrix approach simplifies the computer program since much of the procedure is carried implicitly with the matrices and transformations. The first matrix relates the five tests required to proceed to the correct grouping of organisms to the possible groups of organisms. Hence, this first matrix also contains the information about which tests to select from the data in order to determine the group to which an unknown belongs. Given an unknown, the appropriate transformation is applied to <u>this</u> data to yield +1, -1, or 99, and the result compared with the matrix to determine which organism grouping will be appropriate. Once the organism group for an unknown has been determined, the matrix for that grouping is selected. This matrix again determines the relevant test data for that grouping. A transformation is applied to this data and a comparison is made with the matrix to determine the organism classification. Thus the program simply accepts a data card and, starting with the first matrix, iterates through matrices and transformations until an identification is obtained.

This will be described by using the scheme associated with the organism group most frequently encountered on Apollo spacecraft, namely the Baird-Parker scheme for classifying the two genera Staphylococcus and Micrococcus. As formulated by Baird-Parker the scheme is as follows.

TABLE 1. Diagnostic scheme for classifying staphylococci and micrococci

	Group I *Staphylococcus* Rosenbach						Group II *Micrococcus* Cohn							
Subgroup:	I	II	III	IV	V	VI	1	2	3	4	5	6	7	8
Pink pigment	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Acid from glucose:														
(1) aerobic	+	+	+	+	+	+	+	+	+	+	+	+	±	±
(2) anaerobic	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Coagulase	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Phosphatase	+	+	+	−	−	−	−	−	−	−	−	+	−	−
Acetoin	+	+	−	+	+	+	+	+	+	+	−	−	−	−
Acid from:														
(1) arabinose	−	−	−	−	−	−	−	−	−	+	v	+	−	−
(2) lactose	+	+	v	−	+	v	−	+	v	+	+	+	−	−
(3) maltose	+	+	−	v	+	v	v	+	+	+	+	+	−	±
(4) mannitol	+	−	−	−	−	+	−	−	+	+	+	+	−	−

± = weak or negative, v = variable.

Replacing the microorganism classifications by the number assigned by the PHS, the required tests by the number indicating their position on the PHS data card and the entries by +1, -1, or 99 as appropriate, the scheme (PHS Scheme A) is as follows.

TABLE 2

IDENTIFICATION MATRIX 2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
14	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1
37	1	1	1	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1
42	1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
43	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	1	-1	-1
53	1	1	-1	1	1	1	1	1	1	1	-1	-1	-1	-1
36	-1	-1	-1	-1	-1	-1	-1	-1	-1	1	99	1	-1	-1
38	1	1	99	-1	1	99	-1	1	99	1	1	1	-1	-1
39	1	1	-1	99	1	99	99	1	1	1	1	1	-1	99
40	1	-1	-1	-1	-1	1	-1	-1	1	1	1	1	-1	-1

This is the computerized version of the above table. Next the test results recorded by the PHS must be interpreted as positive, negative or indeterminate. This is done by transforming the entries in the

designated data card columns by the rules needed to make them compatible with the above identification matrix for PHS Scheme A. These rules are shown in Table 3.

TABLE 3

FROM PHS ENTRY TO COMPUTER USAGE

TEST NO.	+1	-1	99
14	6	1,2,3,4,5, 7,8,9	
37	4	3	0,1,2,5
42	2	1	0
43	2	1	0
53	2	1	0
36	3,4	1,2,5	0
38	3,4	1,2,5	0
39	3,4	1,2,5	0
40	3,4	1,2,5	0

The entries in Table 3 are test results as coded on the data card by the PHS. These are converted to +1, -1, or 99 as indicated. Assuming that the computer has read a data card and determined (by Identification Matrix 1) that the colony belongs to Scheme A--microorganisms 1 through 14 in the PHS nomenclature. It then reads matrix 2 (Table 2) and sees that it is concerned with the tests listed on the left hand side, namely, tests 14, 37, ...,40. It then reads those columns on the data card and forms a +1, -1, or 99 according to the rules listed in Table 3. The result is a vector which can be compared with the columns in the Identification matrix (Table 2). If the vector formed from the tests agrees with a column, the organism number of that column is selected as the identification of the colony whose data card is being considered.

Because of the varying test reliability an exact agreement with a column is not always obtained. The "best possible" match (or identification) is then used in keeping with current PHS practice. This practice uses the best of (1) an exact match, (2) one, two, or three single disagreements (i.e., not an exact match, a single test disagreement with one, two, or three organisms--all other classifications having two or more disagreements), or (3) the case where a match is obtained except that two test results are permitted to disagree with those in a single column, all other comparisons being worse. This leads to the possibility of more than one "identification" being made, e.g., where the best vector differs from 2 or 3 organism columns in the matrix each by a single test entry. At present when these multiple possible identities are obtained, all possible identifications of a colony are printed.

Some processed Apollo 13 data is reproduced in Figure 1. The data pertaining to each colony in general consists of three lines. The first line consists of the vector resulting from the five tests used to determine which group of organisms is to be used, the next line contains the vector resulting from the tests pertinent to that grouping, and finally the third line is composed of (i) the sample number and (ii), in the middle of the page, its identification (or multiple identifications) and (iii) at the far right, the identification made by the PHS (printed for comparison purposes here).

An inspection of Figure 1 reveals that there were two colonies where the computer identification did not include that of the PHS, namely, sample numbers D0022 and D0026. Of the 674 colonies sampled on Apollo 13 this occurred 92 times or 13.6 percent of the time. This was considered a disagreement between the computerized scheme and the PHS baseline.

ID	DATE	SN	ORGANISM
VECTOR 1 1 1 -1 1			
VECTOR -1 1 -1 1 1 -1 1 1 -1			
D0001	032770	1	2 002
VECTOR 1 -1 1 -1 1			
D0002	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0003	032770	1	37 037
VECTOR 1 1 1 -1 1			
VECTOR -1 1 -1 1 1 -1 1 1 -1			
D0004	032770	1	2 002
VECTOR 1 -1 -1 -1 1			
D0005	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0006	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0007	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0008	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0009	032770	1	37 037
VECTOR 1 1 1 -1 1			
VECTOR -1 99 -1 -1 -1 -1 -1 -1 -1			
D0010	032770	1	13 013
VECTOR 1 1 1 -1 1			
VECTOR -1 -1 -1 -1 -1 -1 -1 1 -1			
D0011	032770	1	7 13 14 007
VECTOR 1 -1 1 -1 1			
D0012	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0013	032770	1	37 037
VECTOR 1 1 1 -1 1			
VECTOR -1 -1 -1 -1 -1 -1 1 1 -1			
D0014	032770	1	8 11 008
VECTOR 1 -1 1 -1 1			
D0015	032770	1	37 037
VECTOR 1 1 1 -1 1			
VECTOR -1 -1 -1 1 -1 -1 -1 -1 -1			
D0016	032770	1	3 13 013
VECTOR 1 -1 1 -1 1			
D0017	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0018	032770	1	37 037
VECTOR 1 -1 -1 -1 1			
D0020	032770	1	37 037
VECTOR 1 -1 1 -1 1			
D0021	032770	2	37 037
VECTOR 1 1 1 -1 1			
VECTOR -1 99 -1 1 -1 -1 -1 -1 -1			
D0022	032770	2	3 013
VECTOR 1 1 1 -1 1			
VECTOR -1 1 -1 1 1 -1 1 1 -1			
D0023	032770	2	2 002
VECTOR 1 1 1 -1 1			
VECTOR -1 -1 -1 -1 -1 -1 -1 1 -1			
D0024	032770	2	7 13 14 007
VECTOR 1 1 1 -1 1			
VECTOR -1 1 -1 -1 -1 -1 -1 -1 1			
D0025	032770	2	6 006
VECTOR 1 1 1 -1 1			
VECTOR -1 99 -1 -1 1 -1 -1 -1 1			
D0026	032770	2	6 013
VECTOR 1 -1 1 -1 1			

Two Disagreements

Figure 1.

As a comparison this occurred with the previous feasibility program 13.14 percent of the time on Apollo 11.

Of the 92 disagreements of the above form occurring on Apollo 13 data, 60 involved the organism classification category number 13 and of these 60, 28 were a confusion between organism categories 3 and 13. These latter all occurred when an identification had been made on the basis of a single disagreement. Analysis of Table 2 indicates that this probably is due to a weakness or misinterpretation of test number 37, the Baird-Parker glucose (Table 1). It is hoped that this can be corrected to improve the agreement with the PHS.

The program can be written into QUAL so that the identification will be made in that program prior to running QUALSUM in the Lunar Information System. A procedure to choose the one of multiple identifications that the PHS chooses has yet to be added. Continuous refinements can be made simply by modifying the identification system input cards in an attempt to improve the performance--such as eliminating the above confusion between organisms 3 and 13.

Thus, this quarter, a very versatile, and simple, computerized version of the PHS identification scheme has been designed and programmed. It performs as well as the original program demonstrating feasibility of this approach, and it can be readily implemented.

Federal Standard 209a

A. Description. The Sandia Area Office, AEC, requested that Sandia Laboratories again review Federal Standard 209a, "Clean Room and Work Station Requirements, Controlled Environment," to determine whether a revision should be considered.

B. Progress. The need for a revision as well as the advisability of calling a meeting of government agency representatives to consider a revision were discussed. It was the concensus of the Planetary Quarantine Department that the technical content of the standard is sound and that a revision is not warranted at this time. Sandia Laboratories will continue to monitor the application of the standard and will be available for consultation should the need for a revision become evident. A letter to this effect was forwarded to the Sandia Area Office on June 16, 1970. The content of the letter will be furnished the General Services Administration through AEC Headquarters.

Publications

1. D. M. Garst and K. F. Lindell, "Development of Two Closely Controlled Humidity Systems," SC-RR-70-409, June, 1970.

2. W. J. Whitfield, "Electrostatic Deposition Device to Deposit Monolayers of Bacterial Spores on Test Surfaces," SC-R-70-4259, April, 1970.

3. J. P. Brannen, "On the Role of DNA in the Sterilization of Microorganisms," J. Theo. Biol. 27:425 (1970).

4. V. L. Dugan, "A Mathematical Model for the Thermoradiation Inactivation of Dry Bacillus subtilis var. niger Spores," SC-RR-70-203, April, 1970.

5. M. E. Morris and W. J. Whitfield, "Vacuum Probe Sampler," Contamination Control, 27(2), 1970.

6. Contamination Control Handbook. Additional printings of this volume are still being run. The first two printings were made at MSFC, with a total of 1393 copies having been distributed from that source. Subsequent distribution, including three more printings, by the Clearinghouse for Federal Scientific and Technical Information (CFSTI) as of June 1, 1970, amounted to nearly 1100. Since these figures do not include copies distributed by CFSTI after June 1 or copies distributed direct from OTU, NASA Headquarters, it is evident that well over 2500 copies are now in use. Further, it is known that the handbook is being used in Japan and Europe as well as throughout the United States.

Presentations and Briefings

1. Six presentations were made at the Spacecraft Sterilization Technology Seminar held in Atlanta, Georgia, April 15-16.

H. D. Sivinski:	"Bioburden Modeling" "Thermoradiation" "Fine Particle Physics"
R. E. Trujillo:	"Mechanisms of Spore Inactivation"
V. L. Dugan:	"Mathematical Modeling of Thermoradiation Synergism" "Dry-Heat Sterilization Modeling"

2. W. J. Whitfield, "Clean Room Technology," Special Training Session, held in conjunction with the National AACC Meeting, Anaheim, California, April 19.

3. W. J. Whitfield, "Electrostatic Deposition Device to Deposit Monolayers of Bacterial Spores on Test Surface," National AACC Meeting, Anaheim, California, April 22.

4. A briefing on thermoradiation was given Drs. Rotariu and Miller of the Division of Isotopes Development, AEC, by H. D. Sivinski and M. C. Reynolds, in Germantown, Maryland, April 28.

5. A general presentation on thermoradiation was given to representatives from NASA headquarters, NASA centers and major contractors for the Viking program at NASA Headquarters, Washington, D. C. Sandia personnel involved were H. D. Sivinski, M. C. Reynolds, J. A. Hood (Manager, Radiation Effects and Semiconductor Devices Department) and R. M. Jefferson (Supervisor, Reactor Source Applications Division). Given April 29.

6. H. D. Sivinski, "The Importance of Planetary Quarantine," Science Classes, Highland High School, Albuquerque, New Mexico, May 5.

7. H. D. Sivinski, "Planetary Quarantine: Its Nature and Significance," Sigma Xi, New Mexico Institute of Mining and Technology, Socorro, New Mexico, May 27.

8. A general presentation on the nature of thermoradiation was given to representatives from various organizations in the FDA, other HEW branches, the U. S. Army, and NASA. Sandia participants in the presentation were H. D. Sivinski, M. C. Reynolds, V. L. Dugan and R. E. Trujillo. The presentation was made at FDA Headquarters, Washington, D. C. June 2.

9. A briefing on thermoradiation progress was given Dr. Rotariu of the Division of Isotopes Development and Drs. Schulman and Stapleton of the Division of Biology and Medicine, AEC, by H. D. Sivinski, V. L. Dugan, M. C. Reynolds and R. E. Trujillo, Germantown, Maryland, June 2.

10. W. J. Whitfield, Keynote Address, "Contamination Control--A State-of-the-Art Review," First Annual European Contamination Control Symposium, Stuttgart, Germany, June 15-17.

11. H. D. Sivinski, "Planetary Quarantine Research," Kirtland AFB Research Association Meeting, June 23.

Committee Activities

J. P. Brannen, Biomathematics Program Committee, Society for Industrial and Applied Mathematics, National Meeting, Denver, Colorado, June.

W. J. Whitifeld, Chairman, National Particle Monitoring Committee, AACC, National Meeting, Anaheim, California, April 20.

D. M. Garst, Chairman, National Technical Information Committee, AACC, National Meeting, Anaheim, California, April 20.

Patent Activities

The Patent Disclosure filed earlier covering the thermoradiation principles is now under consideration for an AEC patent.

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L. A. Hopkins, Jr., 9500
R. S. Gillespie, 3411
J. L. Gardner, 3422
L. S. Ostrander, 8232

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